

## Inhibition of Membrane Potential-Dependent Amino Acid Transport by Daptomycin

N. E. ALLEN,\* W. E. ALBORN, JR., AND J. N. HOBBS, JR.

*Infectious Disease Research, Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana 46285-0428*

Received 23 April 1991/Accepted 30 September 1991

**Daptomycin inhibits the formation of UDP-*N*-acetylmuramyl-pentapeptide in *Bacillus megaterium* by inhibiting active transport of amino acids incorporated into the pentapeptide. The ability of daptomycin to inhibit active transport and peptidoglycan formation may be due to its ability to disrupt the transmembrane electrochemical gradient.**

Daptomycin (LY146032), a cyclic lipopeptide antibiotic, has diverse effects on growth and metabolism of gram-positive bacteria. A closely related derivative of daptomycin, A21978C<sub>1</sub> (5), was shown to inhibit peptidoglycan formation in *Streptococcus faecalis* without affecting DNA, RNA, or protein synthesis (6). Similar results with daptomycin were reported for *Staphylococcus aureus* (3). In both *S. aureus* and *B. megaterium*, inhibition of peptidoglycan synthesis was predicted to be due to an effect on an early event in the biosynthetic pathway, because the formation of nucleotide-linked sugar-peptide precursors (e.g., UDP-*N*-acetylmuramyl-pentapeptide) needed for peptidoglycan formation was blocked (3). Scanning electron microscopy showed that daptomycin caused gross morphological alterations to the surface of gram-positive cocci (20), which could be related to the permeabilizing effect daptomycin has on the cytoplasmic membrane, which leads to release of intracellular potassium (3). A series of reports (9-11) demonstrated that daptomycin (or closely related congeners) is capable of specific, calcium-dependent interactions with planar bilayer membranes and phospholipid vesicles. Recently, Canepari et al. (4) concluded that the primary target of daptomycin is the biosynthesis of lipoteichoic acid.

Each of the effects reported could be related to the action of daptomycin on the cytoplasmic membranes of gram-positive bacteria (1). As reported elsewhere (2), treatment of *S. aureus* with daptomycin results in a calcium-dependent disruption of membrane energetics; in particular, daptomycin dissipates the membrane or electrical potential ( $\Delta\Psi$  component of the electrochemical gradient). We have attempted to determine whether the effects of daptomycin on cell wall formation are due to a direct effect on peptidoglycan biosynthesis enzymes or to an indirect effect resulting from a disruption of membrane energetics.

**Effects of daptomycin on peptidoglycan biosynthesis enzymes.** Although it was earlier demonstrated that daptomycin inhibited the formation of UDP-*N*-acetylmuramyl-pentapeptide in *B. megaterium* and *S. aureus* (3), a subsequent study (14) provided no evidence for daptomycin-induced accumulation of sugar-peptide precursors occurring earlier in the pathway. We have employed soluble extracts from *B. megaterium* to measure the fosfomycin-sensitive phosphoenolpyruvyl:UDP-*N*-acetylglucosamine-enolpyruvyl transferase reaction (8) by the procedure of Gunetileke and

Anwar (7). Enzyme activity (2.5 pmol/min/mg of extract protein) was completely inhibited by 100  $\mu\text{g}$  of fosfomycin per ml. The same concentration of daptomycin reduced activity by less than 20%.

The study by Mengin-Lecreulx et al. (14) raised the possibility that daptomycin might interfere with one or more of the reactions leading to the formation of UDP-*N*-acetylglucosamine. UDP-*N*-acetylglucosamine pyrophosphorylase catalyzes the conversion of *N*-acetylglucosamine-1-phosphate to UDP-*N*-acetylglucosamine. Using the soluble extracts described above and the chromatographic procedure described by Strominger and Smith (19), we investigated the effects of daptomycin on this reaction. *N*-Acetylglucosamine-1-phosphate in the presence of [<sup>14</sup>C]uridine-5'-triphosphate was converted to the radioactive nucleotide-linked amino sugar at a rate of 703 pmol/min/mg of extract protein. Daptomycin at concentrations of 2 to 50  $\mu\text{g}/\text{ml}$  had no effect on this reaction.

On the basis of the argument that *B. megaterium* synthesizes UDP-*N*-acetylglucosamine by the same pathway as in *Escherichia coli* (i.e., *N*-acetylglucosamine-6P  $\rightarrow$  glucosamine-6P  $\rightarrow$  glucosamine-1P  $\rightarrow$  *N*-acetylglucosamine-1P  $\rightarrow$  UDP-*N*-acetylglucosamine [21]), we used the methodology of Strominger and Smith (19) to further examine soluble extracts for acetyl coenzyme A (CoA)-dependent conversion of glucosamine-1-phosphate, glucosamine-6-phosphate, and *N*-acetylglucosamine-6-phosphate to UDP-*N*-acetylglucosamine. Conversion of glucosamine-1-phosphate in the presence of acetyl-CoA occurred at a rate comparable to that of the pyrophosphorylase reaction (607 pmol/min/mg of extract protein), but acetyl-CoA-dependent conversion of both glucosamine-6-phosphate and *N*-acetylglucosamine-6-phosphate occurred at considerably lower rates (12 and 41 pmol/min/mg of extract protein, respectively). Nevertheless, daptomycin at 2 to 50  $\mu\text{g}/\text{ml}$  did not inhibit any of these reactions. Taken together, the results do not support the notion that daptomycin directly inhibits any of the enzymes involved in peptidoglycan biosynthesis.

**Effects of daptomycin on membrane potential and active transport of amino acids.** Figure 1 demonstrates that daptomycin, in the presence of calcium, dissipates membrane potential ( $\Delta\Psi$ ) in *B. megaterium*. Accumulation of [<sup>14</sup>C]tetraphenylphosphonium bromide was measured by flow dialysis following the procedure exactly as described in a previous report (2), using *B. megaterium* in Mueller-Hinton II broth (containing 40 to 50  $\mu\text{g}$  of calcium per ml; Baltimore Biological Laboratories). The high concentration of dapto-

\* Corresponding author.

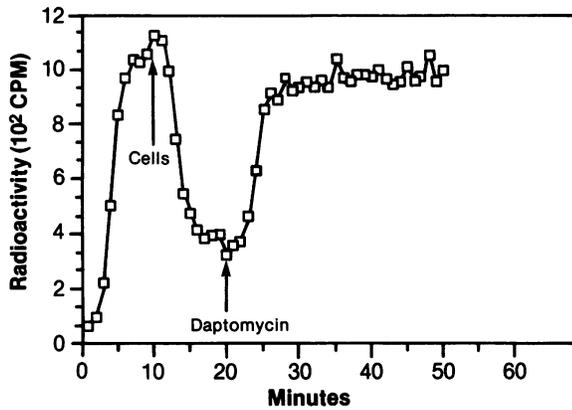


FIG. 1. Effects of daptomycin on accumulation of [ $^{14}\text{C}$ ]tetraphenylphosphonium bromide by *B. megaterium* at pH 7.5. The concentration of daptomycin used was 100  $\mu\text{g/ml}$ . See text and a previous report (2) for description of methods.

mycin was necessary as a result of the high cell concentration required in the flow dialysis experiment. As previously explained (2), the concentration ratio of daptomycin to cells was actually several orders of magnitude less than that encountered at the MIC in a standardized broth susceptibility determination.

The effect on  $\Delta\psi$  suggested to us that the effects of daptomycin on peptidoglycan biosynthesis and, in particular, the inhibition of formation of nucleotide-linked sugar-peptide precursors could result from an effect on membrane energization needed to drive active transport. Active transport was measured as uptake of radiolabeled amino acids by *B. megaterium* in a medium containing the following (in grams per liter): glucose, 2;  $\text{KH}_2\text{PO}_4$ , 6;  $\text{K}_2\text{HPO}_4$ , 6;  $\text{NH}_4\text{Cl}$ , 2;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.05;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.01; chloramphenicol, 0.1; and the radiolabeled amino acid being measured. The medium was then supplemented with 1.25 mM  $\text{CaCl}_2$ , and the pH was adjusted to 6.8. This medium contains chloramphenicol to block amino acid incorporation into protein. It is similar to the medium used in our earlier studies (3) but lacks uracil and two of the three amino acids needed for peptidoglycan biosynthesis. Amino acid uptake in these experiments could be eliminated by lowering the temperature to 4°C, adding 10 mM sodium azide, or omitting glucose from the medium.

The results in Fig. 2 show that 1  $\mu\text{g}$  of daptomycin per ml inhibited uptake of [ $^{14}\text{C}$ ]diaminopimelic acid by approximately 50%. Previous studies have shown that 50% inhibition of peptidoglycan synthesis in *B. megaterium* required 2.5  $\mu\text{g}$  of daptomycin per ml (3). Uptake of L-[ $^{14}\text{C}$ ]alanine and L-[ $^{14}\text{C}$ ]glutamic acid was also inhibited by daptomycin but higher concentrations of antibiotic were required (Fig. 3). Uptake of glutamic acid was stimulated by concentrations of daptomycin up to and including 5  $\mu\text{g/ml}$ . Uptake of all three cell wall amino acids was inhibited 90% or more by 25  $\mu\text{g}$  of daptomycin per ml.

Uptake of each of the cell wall amino acids was completely inhibited by both carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP; 1  $\mu\text{g/ml}$  [5  $\mu\text{M}$ ]) and *N,N'*-dicyclohexylcarbodiimide (DCCD; 20  $\mu\text{g/ml}$  [0.1 mM]), but was unaffected by the addition of 1 mM sodium or potassium arsenate. Assuming that arsenate entered the cells, these effects are consistent with the belief that amino acid transport in *B. megaterium* depends on chemiosmotic energy (18). The

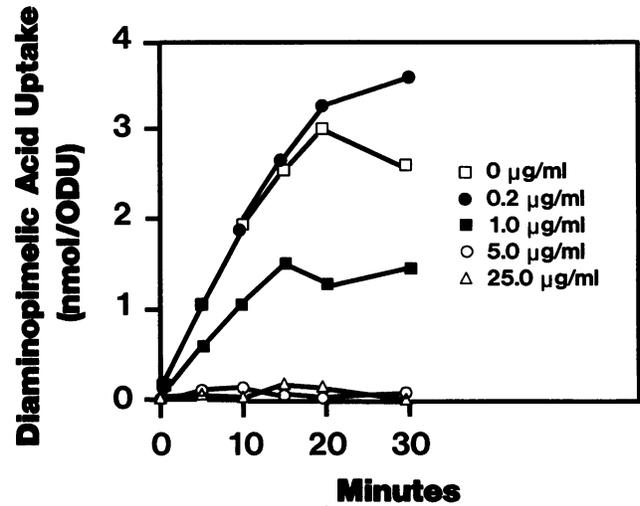


FIG. 2. Effects of daptomycin on uptake of diaminopimelic acid. *B. megaterium* X67 (ATCC 8245) was grown in CGPY broth (12) to mid-log phase, harvested by centrifugation, washed, and resuspended to give a final optical density of 0.5 (measured at 600 nm) in the uptake medium (see text). Diaminopimelic acid uptake in the presence and absence of daptomycin was determined by adding (DL + meso)-2,6-diaminof[ $^{14}\text{C}$ ]pimelic acid (0.5  $\mu\text{Ci/ml}$ ; 25 Ci/mol; final concentration, 20  $\mu\text{M}$ ) to the cell suspension and incubating at 37°C in a shaking water bath. Cell-associated radioactivity was measured by removing aliquots and collecting cells on membrane filters (type HA; pore size, 0.45  $\mu\text{m}$ ; Millipore Corp.). Filters were washed twice with 5 ml of 0.2 M potassium phosphate buffer (pH 7) and counted. Uptake is expressed as concentration per optical density unit (ODU) of cells. The concentrations of daptomycin tested are shown on the figure.

stimulation of glutamic acid uptake by 1 to 5  $\mu\text{g}$  of daptomycin per ml could be related to the apparent specificity of daptomycin for the  $\Delta\psi$  component of the electrochemical gradient (2). If dissipation of  $\Delta\psi$  caused a compensatory

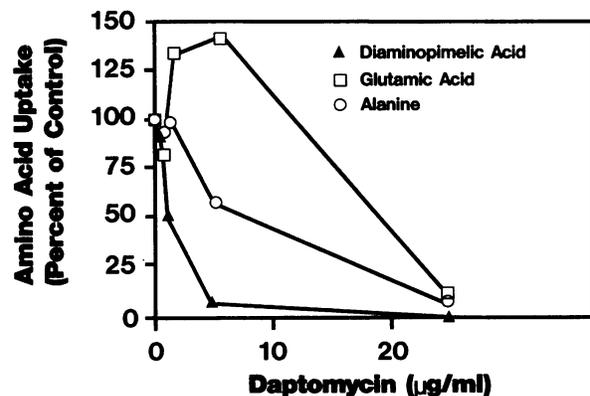


FIG. 3. Effects of daptomycin on uptake of diaminopimelic acid, glutamic acid, and alanine. *B. megaterium* cells were grown and prepared as described in the legend to Fig. 2. Data for 10-min uptake of diaminopimelic acid in the presence and absence of daptomycin were taken from the experiment shown in Fig. 2. The same methodology was used to determine the effects of daptomycin on uptake of L-[ $^{14}\text{C}$ ]glutamic acid (0.02  $\mu\text{Ci/ml}$ ; 1.0 Ci/mol; final concentration, 20  $\mu\text{M}$ ) and L-[ $^{14}\text{C}$ ]alanine (0.5  $\mu\text{Ci/ml}$ ; 2.5 Ci/mol; final concentration, 20  $\mu\text{M}$ ). Uninhibited uptake of diaminopimelic acid, glutamic acid, and alanine during the 10-min incubation was 1.9, 9.8, and 3.7 nmol per optical density unit of cells (measured at 600 nm), respectively.

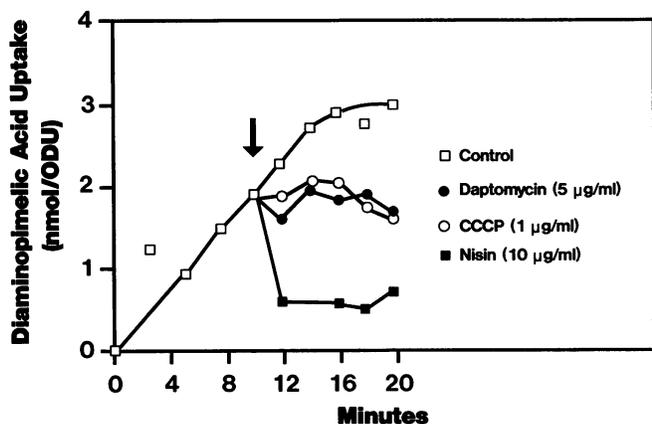


FIG. 4. Effect of daptomycin, nisin, and CCCP on accumulated diaminopimelic acid. *B. megaterium* cells were grown and prepared as described in the legend to Fig. 2. Accumulation of [ $^{14}$ C]diaminopimelic acid (0.1  $\mu$ Ci/ml; 5 Ci/mol; final concentration, 20  $\mu$ M) was monitored for 10 min. At this point, the culture was subdivided, inhibitors were added (indicated by the arrow), and amino acid uptake was monitored for another 10 min. Cell-associated radioactivity was measured as described in the legend to Fig. 2. ODU, optical density unit.

stimulation of  $\Delta$ pH, a concomitant increase in uptake of an anionic substrate (e.g., glutamic acid) might be expected (13).

**Effects of daptomycin on accumulated amino acids.** The effect of daptomycin on the active accumulation of diaminopimelic acid was immediate. Addition of daptomycin (5  $\mu$ g/ml) to cells after they had accumulated [ $^{14}$ C]diaminopimelic acid for 10 minutes resulted in complete cessation of uptake (Fig. 4). The uncoupler CCCP (1  $\mu$ g/ml) had the same effect. Nisin (10  $\mu$ g/ml), a peptide antibiotic which destroys the transmembrane electrochemical gradient in gram-positive bacteria (17), also blocked further uptake. However, nisin caused release of accumulated radioactivity, whereas daptomycin did not. Similarly, daptomycin did not cause a sustained release of accumulated methyl- $\alpha$ -glucopyranoside in *S. aureus* (3).

**Effects of daptomycin on glucose transport.** The experiment shown in Fig. 5 shows that the lowest concentrations of daptomycin that inhibit diaminopimelic acid uptake have no effect on the transport of a nonmetabolizable glucose analog. Glucose is transported in *B. megaterium* by the phosphoenolpyruvate:sugar phosphotransferase system (PTS) (16), which is driven by phosphate bond energy from phosphoenolpyruvic acid. The fact that doses of 1 and 5  $\mu$ g of daptomycin per ml had little effect on the transport of methyl- $\alpha$ -D-[ $^{14}$ C]glucopyranoside argues that daptomycin has a selective action on the electrical potential ( $\Delta$  $\Psi$ ) without directly affecting the PTS or its energy requirements. At 25  $\mu$ g/ml, daptomycin inhibited both amino acid and carbohydrate transport, perhaps as a result of more-complete membrane destruction.

The results from this study in conjunction with those appearing elsewhere (2) indicate that the target site for daptomycin is the energized cytoplasmic membrane. We propose that the effects of daptomycin observed previously on an early step in cell wall biosynthesis (3) result from a calcium-dependent action on the cytoplasmic membrane which dissipates the electrical potential ( $\Delta$  $\Psi$ ). Under conditions where exogenous amino acid availability is limiting for

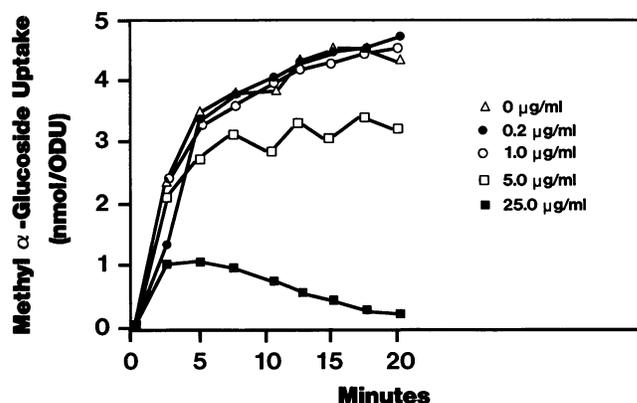


FIG. 5. Effect of daptomycin on PTS. *B. megaterium* cells were grown and prepared as described in the legend to Fig. 2, except that cells were energized for 5 min in the presence of 0.2% glucose, centrifuged, and resuspended in the uptake medium (see text) lacking glucose. Transport of PTS carbohydrates was measured as uptake of methyl- $\alpha$ -D-[ $^{14}$ C]glucopyranoside (0.1  $\mu$ Ci/ml; 5 Ci/mol; final concentration, 20  $\mu$ M). Cell-associated radioactivity was measured as described in the legend to Fig. 2. The daptomycin concentrations tested are shown on the figure. ODU, optical density unit.

peptidoglycan formation (such as the conditions used to measure formation of the nucleotide-linked sugar-peptide precursors [3]), the collapse of  $\Delta$  $\Psi$  by daptomycin would prevent active transport of cell wall amino acids, inhibit formation of sugar-peptide precursors, and shut down the biosynthesis of cell wall peptidoglycan. The collapse of  $\Delta$  $\Psi$  undoubtedly interferes with energy-dependent transport of other nutrients as well (15) and may explain observed effects of daptomycin on other macromolecular biosyntheses in gram-positive bacteria, such as the inhibition of lipoteichoic acid biosynthesis (4).

We thank L. E. Bryan, T. R. Parr, and T. I. Nicas for useful discussions and suggestions.

#### REFERENCES

- Alborn, W. E., Jr., N. E. Allen, J. E. Flokowitsch, and D. A. Preston. 1990. Daptomycin has an effect upon the electrical potential of growing cells of *Staphylococcus aureus*. abstr. 839, p. 226. Program Abstr. 30th Intersci. Conf. Antimicrob. Agents Chemother.
- Alborn, W. E., Jr., N. E. Allen, and D. A. Preston. 1991. Daptomycin disrupts membrane potential in growing *Staphylococcus aureus*. Antimicrob. Agents Chemother. **35**:2282-2287.
- Allen, N. E., J. N. Hobbs, Jr., and W. E. Alborn, Jr. 1987. Inhibition of peptidoglycan biosynthesis in gram-positive bacteria by LY146032. Antimicrob. Agents Chemother. **31**:1093-1099.
- Canepari, P., M. Boaretti, M. del Mar Lleo, and G. Satta. 1990. Lipoteichoic acid as a new target for activity of antibiotics: mode of action of daptomycin (LY146032). Antimicrob. Agents Chemother. **34**:1220-1226.
- Debono, M., M. Barnhart, C. B. Carrell, J. A. Hoffmann, J. L. Occolowitz, B. J. Abbott, D. S. Fukuda, and R. L. Hamill. 1987. A21978C, a complex of new acidic peptide antibiotics: isolation, chemistry, and mass spectral structure elucidation. J. Antibiot. **40**:761-777.
- Eliopoulos, G. M., C. Chauvin, B. Gerson, and R. C. Moellering, Jr. 1985. In vitro activity and mechanism of action of A21978C<sub>1</sub>, a novel cyclic lipopeptide antibiotic. Antimicrob. Agents Chemother. **27**:357-362.
- Gunetileke, K. G., and R. A. Anwar. 1968. Biosynthesis of uridine diphospho-*N*-acetylmuramic acid. II. Purification and

- properties of pyruvate-uridine diphospho-*N*-acetylglucosamine transferase and characterization of uridine diphospho-*N*-acetyl-*enol*pyruvylglucosamine. *J. Biol. Chem.* **243**:5770–5778.
8. Kahan, F. M., J. S. Kahan, P. J. Cassidy, and H. Kropp. 1974. The mechanism of action of fosfomicin (phosphonomycin). *Ann. N.Y. Acad. Sci.* **235**:364–386.
  9. Lakey, J. H., and E. J. A. Lea. 1986. The role of acyl chain character and other determinants on the bilayer activity of A21978C, an acidic lipopeptide antibiotic. *Biochim. Biophys. Acta* **859**:219–226.
  10. Lakey, J. H., R. Maget-Dana, and M. Ptak. 1989. The lipopeptide antibiotic A21978C has a specific interaction with DMPC only in the presence of calcium ions. *Biochim. Biophys. Acta* **985**:60–66.
  11. Lakey, J. H., and M. Ptak. 1988. Fluorescence indicates a calcium-dependent interaction between the lipopeptide antibiotic LY146032 and phospholipid membranes. *Biochemistry* **27**:4639–4645.
  12. Lugtenberg, E. J. J., and P. G. DeHaan. 1971. A simple method for following the fate of alanine-containing components in murein synthesis in *Escherichia coli*. *Antonie van Leeuwenhoek J. Microbiol. Serol.* **37**:537–552.
  13. Mates, S. M., E. S. Eisenberg, L. J. Mandel, L. Patel, H. R. Kaback, and M. H. Miller. 1982. Membrane potential and gentamicin uptake in *Staphylococcus aureus*. *Proc. Natl. Acad. Sci. USA* **79**:6693–6697.
  14. Mengin-Lecreulx, D., N. E. Allen, J. N. Hobbs, and J. van Heijenoort. 1990. Inhibition of peptidoglycan biosynthesis in *Bacillus megaterium* by daptomycin. *FEMS Microbiol. Lett.* **69**:245–248.
  15. Ramos, S., and H. R. Kaback. 1977. The relationship between the electrochemical proton gradient and active transport in *Escherichia coli* membrane vesicles. *Biochemistry* **16**:854–859.
  16. Romano, A. H., S. J. Eberhard, S. L. Dingle, and T. D. McDowell. 1970. Distribution of the phosphoenolpyruvate:glucose phosphotransferase system in bacteria. *J. Bacteriol.* **104**:808–813.
  17. Ruhr, E., and H.-G. Sahl. 1985. Mode of action of the peptide antibiotic nisin and influence on the membrane potential of whole cells and on cytoplasmic and artificial membrane vesicles. *Antimicrob. Agents Chemother.* **27**:841–845.
  18. Strobel, H. J., J. B. Russell, A. J. M. Driessen, and W. N. Konings. 1989. Transport of amino acids in *Lactobacillus casei* by proton-motive-force-dependent and non-proton-motive-force-dependent mechanisms. *J. Bacteriol.* **171**:280–284.
  19. Strominger, J. L., and M. S. Smith. 1959. Uridine diphosphoacetylglucosamine pyrophosphorylase. *J. Biol. Chem.* **234**:1822–1827.
  20. Wale, L. J., A. P. Shelton, and D. Greenwood. 1989. Scanning electron microscopy of *Staphylococcus aureus* and *Enterococcus faecalis* exposed to daptomycin. *J. Med. Microbiol.* **30**:45–49.
  21. White, R. J. 1968. Control of amino sugar metabolism in *Escherichia coli* and isolation of mutants unable to degrade amino sugars. *Biochem. J.* **106**:847–858.