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Additional Antibiotic Inhibitors of Peptidoglycan Synthesis

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Diumycin, janiemycin, nisin, and subtilin inhibited peptidoglycan synthesis catalyzed by particulate enzyme systems from *Bacillus stearothermophilus* and *Escherichia coli*. All of these, except for nisin, also induced accumulation of the lipid intermediate in peptidoglycan synthesis. Concentrations required for 50% inhibition of peptidoglycan synthesis were less than 0.1 μg/ml for diumycin and in the range of 10 to 100 μg/ml for janiemycin, nisin, and subtilin in both organisms. The discrepancy between the extremely low concentration of diumycin required to inhibit the in vitro system from *E. coli* and the much higher concentration required to inhibit growth of the organism is noteworthy.

The first membrane-bound reaction of peptidoglycan synthesis in gram-negative and gram-positive organisms is the reaction of the soluble precursor, uridine-diphospho-*N*-acetylmuramyl-L-alanyl-D-γ-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanyl-D-alanine (UDP-MurNAc-Ala-Glu-Dap-Ala-Ala), with a C_{55}-isoprenyl phosphate to give the membrane-bound intermediate, MurNAc (pentapeptide)-P-P-lipid. UDP-*N*-acetylglucosamine (UDP-GlcNAc) then transfers GlcNAc to give GlcNAc-MurNAc (pentapeptide)-P-P-lipid. The modified disaccharide-peptide unit is then transferred to an acceptor with release of C_{55}-isoprenyl pyrophosphate, which is dephosphorylated to start a new cycle of synthesis. The peptide moiety of this intermediate can then be further modified, e.g., by amidation or by addition of the amino acids of the interpeptide bridge. The resulting oligosaccharide chains are finally modified by transpeptidation to cross-link a portion of the peptide chains, and by D-alanine carboxypeptidase. This yields the final cross-linked cell wall material.

Antibiotics are known which interfere with these pathways at three different places. Vancomycin, ristocetin, ristomycetin (2, 28), enduracidin (17), moenomycin, prasinomycin, 11,837 RP (14), macarbomycin (29), and several detergents such as deoxycholate and *n*-octanol (16) appear to inhibit the transfer of the disaccharide-peptide unit from the lipid intermediate to the acceptor. Bacitracin inhibits the dephosphorylation of C_{55}-isoprenyl pyrophosphate (25). The penicillins and cephalosporins inhibit transpeptidase and carboxypeptidase activity (2, 28).

In this investigation, the effects of several lipid-containing antibiotics, diumycin (19), moenomycin (33), prasinomycin (34), and janiemycin (21), as well as the peptide antibiotics nisin (18) and subtilin (9), on peptidoglycan synthesis catalyzed by particulate enzyme preparations from *Bacillus stearothermophilus* and *Escherichia coli* were studied. While this work was in progress, the effects of two of these, prasinomycin and moenomycin, were also studied by Lugtenberg et al. (14). Similarities exist among diumycin, macarbomycin, moenomycin, prasinomycin, and 11,837 RP (6, 15, 20, 27, 29; F. L. Weisenborn, personal communication), between enduracidin and janiemycin (21), and between nisin and subtilin (3, 4). In vivo, diumycin (12), enduracidin (31), macarbomycin (29), moenomycin (5), prasinomycin (13), and janiemycin (A. L. Laskin and W. M. Chan, personal communication) have been shown to cause accumulation of the uridine nucleotide cell wall precursors in *Staphylococcus aureus*, as had been found with bacitracin, vancomycin, ristocetin, penicillins, and cephalosporins (2, 28).

MATERIALS AND METHODS

Preparation of particulate enzymes. *B. stearothermophilus* ATCC 15952 was grown on 10 g of tryptone, 5 g of yeast extract, 1 g of glucose, 3 g of K_2HPO_4, 1 g of KH_2PO_4, 3 g of NaCl, and 1 ml of

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silicone antifoam A (Dow Chemical Corp., Midland, Mich.) per liter of deionized water at 60 C with vigorous aeration. The cells were harvested in the logarithmic phase, washed with 50 mM tris-(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 8.5, containing 10 mM $MgCl_2$. Cells were broken in a Mini-mill (Gifford-Wood, Hudson, N.Y.) with glass beads, and the membrane fraction was suspended in Tris-hydrochloride-$MgCl_2$ buffer at a concentration of 17 μg of protein/ml, as described previously for *B. megaterium* (35).

The particulate enzyme from *E. coli* H 102 was a kind gift from T. Kamiryo. It was prepared by alumina grinding (1, 7, 8) and suspended in 50 mM Tris-hydrochloride buffer, pH 8.0, and 10 mM $MgCl_2$ to give about 30 mg of protein/ml.

Peptidoglycan synthesis. The assay mixture for the *B. stearothermophilus* system contained 0.2 M Tris-hydrochloride buffer, pH 8.5, 10 mM $MgCl_2$, 0.25 mM UDP-MurNAc-Ala-Glu-Dap-Ala-Ala, 0.27 mM UDP-[^{14}C]GlcNAc (2.6 mCi/mmol; about 16,000 counts/min), and 67 μg of particulate enzyme protein in a total volume of 20 μliters. After incubation for 30 min at 37 C, the reaction was stopped by the addition of 10 μliters of isobutyric acid-1N NH_4OH (5:3) and cooled to 0 C. The entire reaction mixture plus washings was spotted on a 1-cm line at the origin of a sheet of Whatman 3MM paper which was then developed by descending chromatography with isobutyric acid-0.125 N NH_4OH (5:3). Chromatograms were autoradiographed for 4 days with Kodak RB 54 X-ray film. Areas corresponding to peptidoglycan, UDP-GlcNAc, and GlcNAc-MurNAc (pentapeptide)-P-P-lipid were cut from the paper and counted in 15 ml of toluene-based scintillation fluid. Under these conditions, about 2,400 counts/min were found in peptidoglycan at the origin and about 140 counts/min in the lipid intermediate, with blank values (obtained by omission of UDP-MurNAc-pentapeptide) of about 100 and 90 counts/min, respectively, subtracted.

The assay mixture for the *E. coli* system contained 0.2 M Tris-hydrochloride buffer, pH 8.0, 20 mM $MgCl_2$, 0.25 mM UDP-MurNAc-Ala-Glu-Dap-Ala-Ala, 16 μM UDP-[^{14}C]GlcNAc (42 mCi/mmol; about 16,000 counts/min), and 90 μg of particulate enzyme protein in a total volume of 20 μliters. All other details were as for the *B. stearothermophilus* system. Under these conditions, about 3,800 counts/min were found at the origin and about 600 to 1,600 counts/min in the lipid intermediate, with blank values of about 200 and 150 counts/min, respectively, subtracted.

Radiochemicals. UDP-[^{14}C]GlcNAc was supplied by New England Nuclear Corp., Boston, Mass. (specific activity, 42 mCi/mmol).

Antibiotics. Diumycin, prasinomycin, janiemycin, and subtilin were generous gifts of the Squibb Institute for Medical Research, Princeton, N.J. Moenomycin was kindly given by Hoechst Pharmaceutical Co., Frankfurt, Germany, and highly purified samples of both subtilin and nisin were generously supplied by Erhard Gross, National Institutes of Health.

RESULTS

Systems for peptidoglycan synthesis in vitro. Particulate enzyme preparations were prepared from *B. stearothermophilus* vegetative cells in a Mini-mill (24, 35) and from *E. coli* by alumina grinding (1, 7, 8). Such preparations catalyze the synthesis of cross-linked peptidoglycan in *B. stearothermophilus* (P. E. Linnett and J. L. Strominger, in preparation) and in *E. coli* (1, 7, 8) from UDP-MurNAc-pentapeptide and UDP-GlcNAc. Formation of peptidoglycan and of the lipid intermediate (GlcNAc-MurNAc [pentapeptide]-P-P-lipid) was followed by measuring the incorporation of [^{14}C]GlcNAc from UDP-[^{14}C]GlcNAc dependent on unlabeled UDP-MurNAc-pentapeptide into material at the origin and at R_f about 0.9, respectively, of paper chromatograms developed in isobutyric acid-0.125 N NH_4OH (5:3). The only other radioactive areas corresponded to unchanged UDP-GlcNAc (R_f 0.10) together with small amounts of GlcNAc-1-P (R_f 0.26) and GlcNAc (R_f 0.57).

Inhibition by antibiotics. Antibiotics were added as aqueous solutions to the peptidoglycan synthesis systems outlined above. The results are shown for a variety of concentrations of diumycin, moenomycin, prasinomycin, janiemycin, nisin and subtilin in Fig. 1-3, and antibiotic concentrations giving 50% inhibition of peptidoglycan synthesis are summarized in Table 1. The antibiotics fell into two classes with regard to their level of effectiveness against peptidoglycan synthesis in vitro. Moenomycin, prasinomycin, and diumycin showed 50% inhibition of peptidoglycan synthesis at concentrations less than 0.1 μg/ml with enzyme preparations from both *B. stearothermophilus* and *E. coli*, whereas the 50% inhibitory concentrations for janiemycin, nisin, and subtilin were 20 to 100 μg/ml. Accumulation of the lipid intermediate was found with all of the antibiotics tested except nisin at concentrations at which peptidoglycan synthesis was inhibited with enzyme preparations from both organisms. The radioactivity found in the lipid intermediate was very low for the *B. stearothermophilus* system, but the accumulation with antibiotics was reproducible and was supported by the more significant data obtained with the *E. coli* system.

Derivatives of diumycin. Diumycin can be separated into four components, diumycins A, A′, B, and B′ (20; W. Slusarchyk and F. Weisenborn, personal communication). The activities of these components were compared in the cell-free peptidoglycan synthesis system

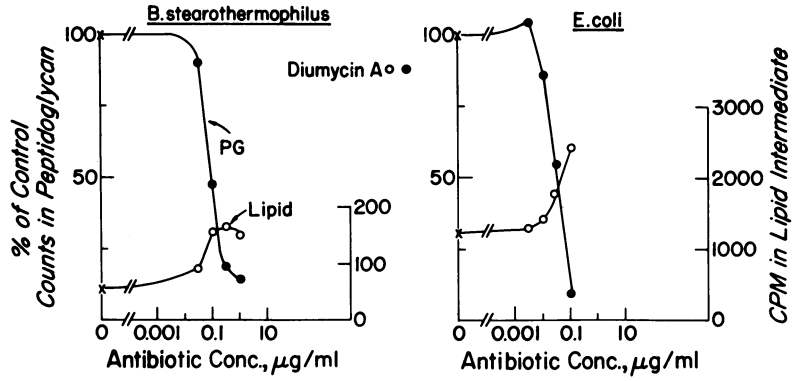

FIG. 1. *Inhibition of peptidoglycan synthesis and accumulation of lipid intermediate by diumycin A in cell-free systems from B. stearothermophilus and E. coli.* (●) *Peptidoglycan synthesis;* (○) *lipid intermediate synthesis.*

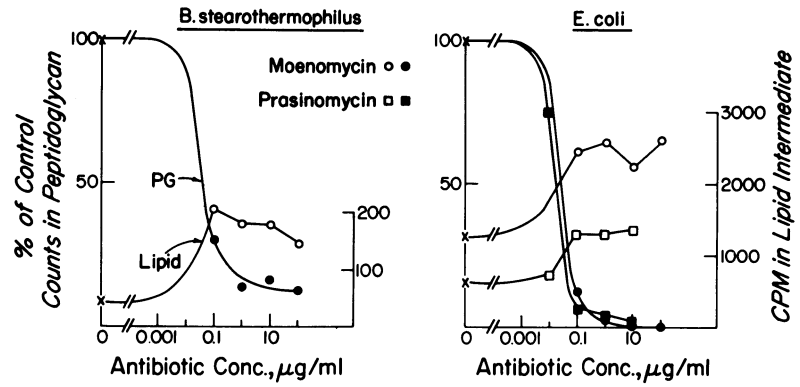

FIG. 2. *Inhibition of peptidoglycan synthesis and accumulation of lipid intermediate by moenomycin and prasinomycin A in cell-free systems from B. stearothermophilus and E. coli. Moenomycin:* (●) *peptidoglycan synthesis;* (○) *lipid intermediate synthesis. Prasinomycin A:* (■) *peptidoglycan synthesis;* (□) *lipid intermediate synthesis.*

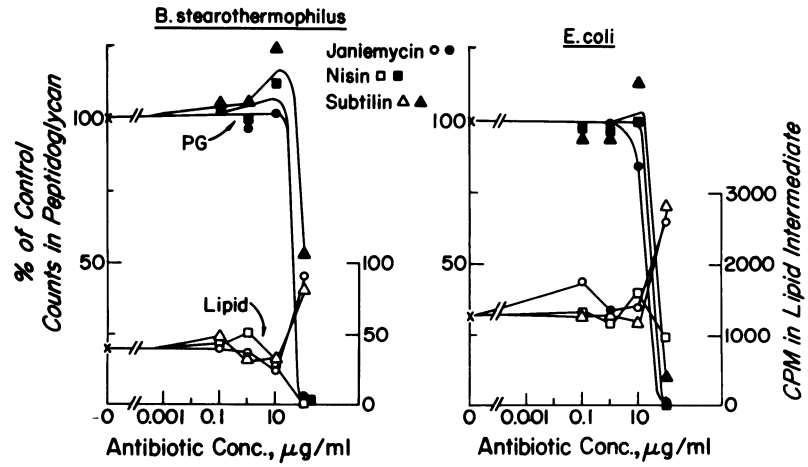

FIG. 3. *Inhibition of peptidoglycan synthesis and accumulation of lipid intermediate induced by janiemycin, nisin, and subtilin in cell-free systems from B. stearothermophilus and E. coli. Inhibition of peptidoglycan synthesis:* (●) *janiemycin;* (■) *nisin;* (▲) *subtilin. Lipid intermediate synthesis:* (○) *janiemycin;* (□) *nisin;* (△) *subtilin.*

from *E. coli*. Each component caused 50% inhibition of peptidoglycan synthesis at concentrations of 0.01 to 0.02 μg/ml and caused accumulation of the lipid intermediate (Fig. 4).

DISCUSSION

In this paper, several additional antibiotic inhibitors of peptidoglycan synthesis are described which were active against preparations from both the gram-negative organism *E. coli* and the gram-positive organism *B. stearothermophilus*, viz., diumycin, janiemycin, nisin, and subtilin. In addition, the inhibitory effects of moenomycin and prasinomycin (14) were confirmed. It is of interest that the *E. coli* cell-free system is as sensitive to diumycin, moenomycin, and prasinomycin as is that from *B. stearothermophilus*, since in vivo *E. coli* and other gram-negative bacteria are resistant to these antibiotics (19, 32, 34). These data suggest that the relative resistance of the gram-negative organisms must be due to a permeability barrier. The low concentrations at which diumycin, moenomycin, and prasinomycin inhibit peptidoglycan synthesis in vitro (~0.05 μg/ml) make this site a good candidate for the killing site in vivo and are noteworthy because only penicillins are able to inhibit peptidoglycan synthesis in vitro at such exceedingly low concentrations (7, 8). Since all of the antibiotics studied here except for nisin induced accumulation of GlcNAc-MurNAc (pentapeptide)-P-P-lipid, the inhibited step or steps of peptidoglycan synthesis must be the step in which this intermediate is utilized for peptidoglycan synthesis. Nisin inhibited synthesis of both lipid intermediate and peptidoglycan. There is no previous example of an antibiotic which does that, although some detergents do (16). Nisin could be an inhibitor of one of the early steps in peptidoglycan synthesis.

TABLE 1. *Antibiotic concentrations required for 50% inhibition of peptidoglycan synthesis, and accumulation of lipid intermediate in vitro*

Antibiotic	Concn for 50% inhibition of peptidoglycan synthesis[a] (μg/ml)		Accumulation of lipid intermediate[b]	
	B. stearothermophilus	*E. coli*	*B. stearothermophilus*	*E. coli*
Diumycin A	0.09	0.04	+	+
Moenomycin	0.04	0.02	+	+
Prasinomycin A ..		0.02		+
Janiemycin	40	20	+	+
Nisin	40	40	−	−
Subtilin	100	50	+	+

[a] Estimated from the data shown in Fig. 1-3.

[b] Plus represents accumulation of GlcNAc-MurNAc (pentapeptide)-P-P-C_{55} lipid in the presence of concentrations of the antibiotic which inhibit peptidoglycan synthesis.

The nonaggregated molecular weights of diumycin A and B, moenomycin, and prasinomycin lie in the range of 1,600 to 2,300 (6, 10, 11). The concentration of 0.1 μg/ml, at which peptidoglycan synthesis is completely inhibited by these antibiotics, is approximately 50 nM. Since this concentration is at least two orders of magnitude less than that of the UDP-[^{14}C]GlcNAc substrate (16 μM in the *E. coli* assay system and 260 μM in the *B. stearothermophilus* assay) and of the UDP-MurNAc-pentapeptide substrate (250 μM in both assays), an inhibitory mechanism in which these antibiotics act by stoichiometric binding

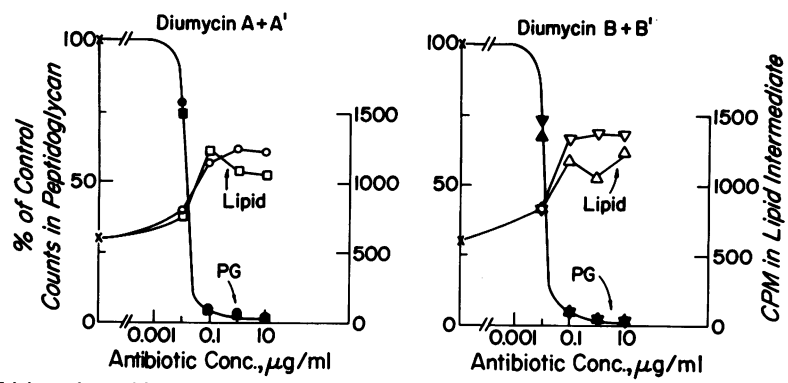

FIG. 4. *Inhibition of peptidoglycan synthesis and accumulation of lipid intermediate by diumycins A, A', B, and B' in cell-free system from E. coli. Inhibition of peptidoglycan synthesis:* (●) *diumycin A;* (■) *A';* (▲) *B;* (▼) *B'. Lipid intermediate synthesis:* (○) *diumycin A;* (□) *A';* (△) *B;* (▽) *B'.*

to the D-alanyl D-alanine carboxy terminus of the lipid intermediate, as has been suggested for vancomycin, ristocetin, and ristomycetin (2, 23), appears to be unlikely. In fact, the structures of the lipid moieties of diumycins and related antibiotics (22, 26, 27, 30) suggest that they are derived from C_{25}-isoprenyl alcohols, and these antibiotics also contain phosphate. They could conceivably, therefore, function as analogues of C_{55}-isoprenyl phosphate-containing lipids which function in peptidoglycan synthesis (28).

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ADDENDUM

After this paper was submitted for publication, a recent report of the effects of diumycin A on peptidoglycan synthesis was brought to our attention (E. J. J. Lugtenberg, J. A. Hellings, and G. J. van de Berg, Inhibition of peptidoglycan synthesis by the antibiotic diumycin A, Antimicrob. Ag. Chemother. **2**:485-491, 1972). The composition of diumycins A, A', B, and B' has also been reported recently (W. A. Slusarchyr, J. L. Bouchard-Ewing, and F. L. Weisenborn, Diumycin A' and B', new members of the diumycin family of antibiotics, J. Antibiot. (Tokyo) **26**: 391-393, 1973.

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