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Katanosin B and Plusbacin A_3, Inhibitors of Peptidoglycan Synthesis in Methicillin-Resistant *Staphylococcus aureus*

HIDEKI MAKI,* KENJI MIURA, AND YOSHINORI YAMANO

Discovery Research Laboratories, Shionogi & Co., Ltd., Osaka, Japan

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Both katanosin B and plusbacin A_3 are naturally occurring cyclic depsipeptide antibiotics containing a lactone linkage. They showed strong antibacterial activity against methicillin-resistant *Staphylococcus aureus* and VanA-type vancomycin-resistant enterococci, with MICs ranging from 0.39 to 3.13 μg/ml, as well as against other gram-positive bacteria. They inhibited the incorporation of *N*-acetylglucosamine, a precursor of cell wall synthesis, into peptidoglycan of *S. aureus* whole cells at concentrations close to their MICs. In vitro studies with a wall-membrane particulate fraction of *S. aureus* showed that katanosin B and plusbacin A_3 inhibited the formation of lipid intermediates, with 50% inhibitory concentrations (IC_{50}s) of 2.2 and 2.3 μg/ml, respectively, and inhibited the formation of nascent peptidoglycan, with IC_{50}s of 0.8 and 0.4 μg/ml, respectively. Vancomycin, a well-known inhibitor of transglycosylation, did not inhibit the formation of lipid intermediates but did inhibit the formation of nascent peptidoglycan, with an IC_{50} of 4.1 μg/ml. Acetyl-Lys-D-Ala-D-Ala, an analog of the terminus of the lipid intermediates, effectively suppressed the inhibition of transglycosylation by vancomycin, but did not suppress those by katanosin B and plusbacin A_3. These results indicate that the antibacterial activity of katanosin B and plusbacin A_3 is due to blocking of transglycosylation and its foregoing steps of cell wall peptidoglycan synthesis via a mechanism differing from that of vancomycin.

Katanosin B and plusbacin A_3 were previously isolated from a strain related to the genus *Cytophaga* and a strain of *Pseudomonas*, respectively (35, 36). They exhibited in vitro activity against gram-positive bacteria and showed therapeutic effects in mice infected with *Staphylococcus aureus* by subcutaneous administration (35, 36). Structure analysis revealed them both to be cyclic depsipeptide antibiotics containing a lactone linkage, although their amino acid residues and sequences were very different from each other (17, 37) (Fig. 1). They were reported to be inhibitors of cell wall synthesis, judging from their inhibition of radio-labeled diaminopimelic acid incorporation into the cell wall peptidoglycan of a *Bacillus* strain.

Vancomycin has been used to treat gram-positive bacterial infection and is regarded as a last resort for the treatment of methicillin-resistant *S. aureus* infection. However, the prevalence of vancomycin-resistant enterococci has already become an important problem (21, 26), and even the emergence of *S. aureus* clinical isolates with reduced vancomycin susceptibility has been reported recently (6, 11, 12, 34, 38, 39). Thus, novel drugs to replace vancomycin are urgently required. Drugs with different modes of action from vancomycin should be promising candidates against vancomycin-resistant strains. In this study, the mechanism of action of katanosin B and plusbacin A_3 was studied using target organisms with vancomycin for comparison.

MATERIALS AND METHODS

Bacteria and growth conditions. The organisms used in this experiment are listed in Table 1. Unless otherwise stated, *S. aureus* strains were grown in tryptic soy broth (TSB; Difco Laboratories, Detroit, Mich.) at 37°C with aeration.

* Corresponding author. Mailing address: Discovery Research Laboratories, Shionogi & Co., Ltd., 3-1-1, Futaba-Cho, Toyonaka, Osaka 561-0825, Japan. Phone: 81-6-6331-8081. Fax: 81-6-6331-8612. E-mail: hideki.maki@shionogi.co.jp

Drugs. Katanosin B and plusbacin A_3 were isolated as described previously (35, 36). Vancomycin is commercially available (Shionogi, Osaka, Japan).

Susceptibility test. MICs were determined by using serial twofold microdilutions of antibiotic in cation-adjusted Mueller-Hinton broth (Difco Laboratories) (28). The overnight culture of bacteria was inoculated at 5×10^5 CFU/ml and incubated at 35°C for 20 h before the MIC was scored.

Incorporation of ^{14}C-labeled *N*-acetylglucosamine into cell wall peptidoglycan. *S. aureus* NCTC8325 was cultivated at 37°C overnight in CGPY broth (Na_2HPO_4, 6 g; NaCl, 3 g; $MgCl_2 \cdot 6H_2O$, 0.1 g; NH_4Cl, 2 g; Na_2SO_4, 0.15 g; KH_2PO_4, 3 g; Bactopeptone, 10 g; yeast extract, 0.1 g; and glucose, 5 g [per liter], pH 7.0) (19). The culture was diluted 100-fold with the same medium and further cultivated until an optical density at 660 nm (OD_{660}) of 0.2 was reached by monitoring the density with a Spectronic 20A spectrophotometer (Shimazu, Kyoto, Japan). The cells were pelleted by centrifugation at $8{,}000 \times g$ for 10 min and resuspended in modified cell wall synthesis medium (for [^{14}C]GlcNAc incorporation experiment, KH_2PO_4 [6 g], K_2HPO_4 [6 g], NH_4Cl [2 g], $MgSO_4 \cdot 7H_2O$ [5 mg], $FeSO_4$ [5 mg], glucose [100 mg], uracil [40 mg], L-alanine [50 mg], L-glutamic acid [120 mg], L-lysine [50 mg], chloramphenicol [100 mg] [per liter]) to an OD_{660} of 0.1 (19, 23). One 1-ml portion of the cell suspension containing each concentration of the drug and 5 μM [^{14}C]GlcNAc (1.85 GBq/mmol; Amersham Pharmacia Biotech UK Limited, Buckinghamshire, England) was incubated at 37°C with aeration. After 30 min of incubation, a 0.5-ml portion of the cell suspension was transferred to a microcentrifuge tube containing 0.5 ml of ice-cold 10% trichloroacetic acid (TCA). The mixture was incubated at 90°C for 15 min, placed on ice for 30 min, and filtered with a membrane filter (type HA; pore size, 0.45 μm; diameter, 25 mm; Millipore Corp., Bedford, Mass.) followed by 5% TCA washing. The membrane filter was then immersed in 5 ml of Pico-Fluor 40 (Packard Instrument Company, Meriden, Conn.), and the radioactivity was counted with a liquid scintillation analyzer, Tri-Carb 2000CA (Packard Instrument Company).

Preparation of wall-membrane particulate and supernatant fraction. The overnight culture of *S. aureus* SRM133 was diluted 50-fold in warm TSB and further cultivated at 37°C with shaking to the logarithmic phase of growth. Next, cells were harvested and washed with cold 0.05 M Tris-HCl buffer (pH 8.0) containing 0.1 mM $MgCl_2$ (buffer A). The cells were resuspended in buffer A and disrupted with glass beads in a mechanical cell homogenizer, HOM-MSK (B. Braun Biotech Inc., Allentown, Pa). A wall-membrane particulate fraction was prepared by differential centrifugation between $8{,}000 \times g$ for 10 min and $100{,}000 \times g$ for 30 min. The pellet was washed once with buffer A, resuspended in it, and stored at −80°C. For preparation of a supernatant fraction, *S. aureus* SRM133 cells grown overnight were harvested and disrupted as described above. The supernatant after differential centrifugation was collected and stored as

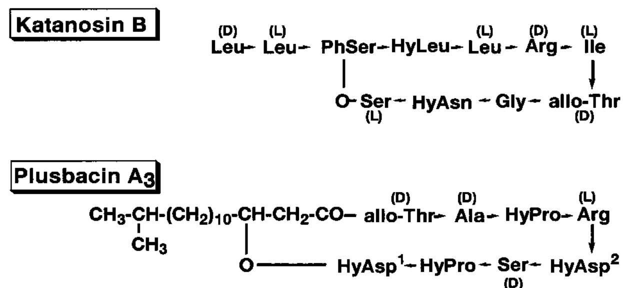

FIG. 1. Structures of katanosin B and plusbacin A_3. PhSer, L-*threo*-β-phenylserine; HyLeu, L-*threo*-β-hydroxyleucine; HyAsn, L-*threo*-β-hydroxyasparagine; HyAsp[1], L-*threo*-β-hydroxyaspartic acid; HyAsp[2], D-*threo*-β-hydroxyaspartic acid; HyPro, L-*trans*-3-hydroxyproline.

supA at −80°C. The protein contents of a wall-membrane particulate fraction and supA were determined by using the Bio-Rad Dc protein assay reagent (Bio-Rad Laboratories, Hercules, Calif.) with bovine serum albumin as the standard.

Enzymatic reactions. Enzymatic syntheses of lipid intermediates and nascent peptidoglycan were detected with labeled glycine. The reaction mixture contained 60 mM Tris-HCl (pH 8.5), 30 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 330 μM UDP-GlcNAc, 1.7 mM ATP, 0.5 mg of protein/ml of supA, 7.1 μM [^{14}C]glycine (4.17 GBq/mmol; NEN Life Science Products, Inc. Boston, Mass.), and 0.5 mg of a wall-membrane particulate fraction per ml. If necessary, each concentration of the drugs was added to the reaction mixture. The reaction was performed at 30°C for 60 min. The reaction mixture was then spotted onto a cellulose thin-layer chromatography (TLC) plate (TLC plates, cellulose F precoated; Merck, Darmstadt, Germany) and developed with isobutyric acid–1 M ammonia (5:3) as the solvent for 17 h at room temperature. Radioactivity on the plate was detected with Bio-Imaging Analyzer BAS2000 (Fuji Photo Film Co., Ltd., Tokyo, Japan). For the suppression experiment, each concentration of acetyl-Lys-D-Ala-D-Ala (Sigma Chemical Co., St. Louis, Mo.) was added to the reaction mixture containing the respective drug (12.5 μg of vancomycin 1.56 μg of katanosin B, or 0.78 μg of plusbacin A_3 per ml, at which concentrations the formation of nascent peptidoglycan was almost inhibited).

Suppression of growth inhibition. The overnight culture of *S. aureus* NCTC8325 was streaked over a TSA (Difco) plate with a swab. Paper disks containing 5 μg of each antibiotic were placed on it, and then 50 μg of protein of a wall-membrane particulate was spotted near the each disk. The plate was incubated at 37°C overnight.

RESULTS

Antibacterial activity. MICs of katanosin B, plusbacin A_3, and vancomycin against representative staphylococci and enterococci are listed in Table 1. Katanosin B and plusbacin A_3 showed strong activity irrespective of methicillin and vancomycin resistance.

Effect on incorporation of [^{14}C]GlcNAc into peptidoglycan of *S. aureus* whole cells. Both katanosin B and plusbacin A_3 strongly inhibited the incorporation of [^{14}C]GlcNAc into staphylococcal cell wall peptidoglycan (Fig. 2A). The degree of inhibition correlated with the concentration of the drug. The 50% inhibitory concentrations (IC_{50}s) of katanosin B, plusbacin A_3, and vancomycin were 0.28, 0.62, and 1.0 μg/ml, respectively, which were all close to their MICs.

Formation of lipid intermediates and nascent peptidoglycan in vitro. Newly formed lipid intermediates and nascent peptidoglycan were separated on TLC and detected by incorporation of [^{14}C]glycine via successive reactions catalyzed by several enzymes, including MraY (phospho-*N*-acetylmuramoyl-pentapeptide translocase) (3, 31), MurG (*N*-acetylglucosaminyl transferase) (22, 24, 41), FemX (the postulated enzyme involved in attachment of the first glycine to the pentaglycine interpeptide, and *fmhB* was recently shown as the strong candidate for its gene) (18, 33, 42), FemA, FemB, and transglycosylase (Fig. 3 and 4). The R_f values of the signals indicated the positions of lipid intermediates and nascent peptidoglycan according to a previous report (30), whereas the signal corresponding to lipid intermediates was obviously not a single band. Although no information is available, the signal might represent a mixture of glycine additives with different numbers of glycine or might represent lipid I and lipid II. Tunicamycin, an MraY inhibitor, inhibited the formation of both lipid intermediates and nascent peptidoglycan simultaneously (data not shown), and vancomycin, a transglycosylase inhibitor, inhibited the formation of only nascent peptidoglycan (as shown later). Lipid intermediates and nascent peptidoglycan were hardly detected in the experiment without UDP-GlcNAc, which proved that we could detect these prod-

TABLE 1. Properties of bacterial strains used in this study

Strain	Relevant phenotype[a]	MIC (μg/ml)			Source[b] or reference
		Katanosin B	Plusbacin A_3	Vancomycin	
Staphylococcus aureus					
NCTC8325	Mc^s	0.39	0.78	1.56	29
Smith (diffuse)	Mc^s	0.39	0.78	0.78	13
SRM133	Mc^r	0.39	0.78	1.56	Laboratory strain
SR3637	Mc^r	0.39	1.56	1.56	Clinical isolate
Mu50	Mc^r Vm^i	0.39	1.56	12.5	12
Enterococcus faecalis					
SR1004	Vm^s	0.78	3.13	1.56	Clinical isolate
SR7914	Vm^r (VanA)	0.78	1.56	>50	Clinical isolate
Enterococcus faecium					
SR15941	Vm^s	0.78	3.13	1.56	Clinical isolate
SR7917	Vm^r (VanA)	0.78	3.13	>50	Clinical isolate

[a] Abbreviations: Mc, methicillin; Vm, vancomycin; s, susceptible; r, resistant; i, intermediate.
[b] Clinical isolates were from Japan.

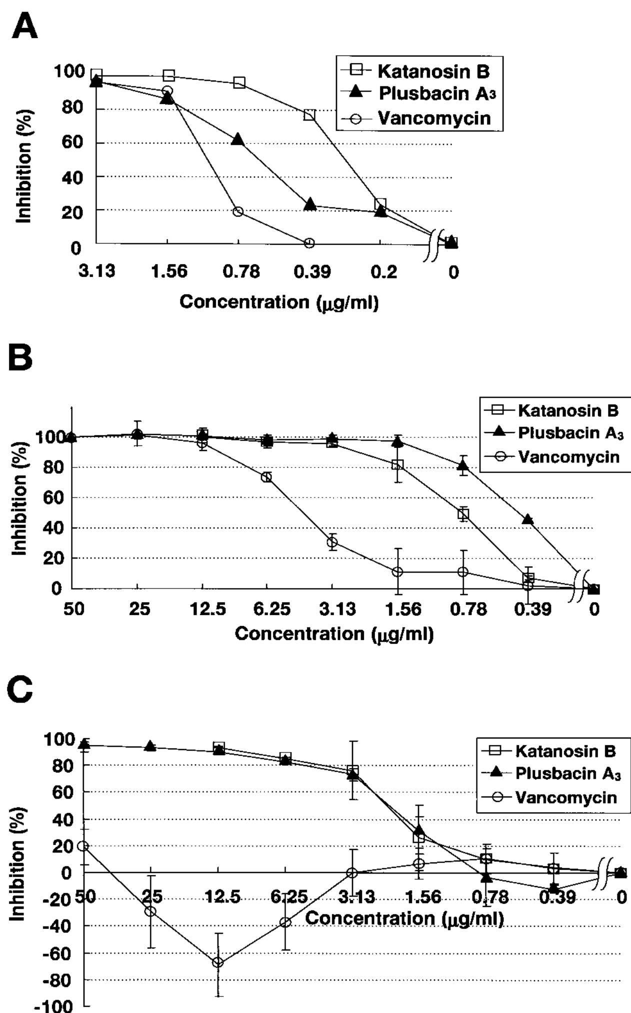

FIG. 2. (A) Inhibition of incorporation of [^{14}C]GlcNAc into peptidoglycan of *S. aureus* whole cells. Assays were carried out as described in Materials and Methods. Radioactivity was measured and expressed as the inhibition rate by comparison with a control without antibiotic. (B and C) Inhibition of formation of (B) lipid intermediates and (C) nascent peptidoglycan. Radioactivity incorporated into each product shown in Fig. 4 was measured and expressed as the inhibition rate by comparison with a control without antibiotic. Results are means ± standard deviations.

ucts through successive reactions from MraY to transglycosylation. Katanosin B and plusbacin A_3 inhibited nascent peptidoglycan formation, with IC_{50}s of 0.8 and 0.4 μg/ml, respectively (Fig. 2B), which were close to the MICs, as well as the case of [^{14}C]GlcNAc incorporation into peptidoglycan of whole cell. Vancomycin inhibited nascent peptidoglycan formation with an IC_{50} of 4.1 μg/ml, which was several times higher than those of katanosin B and plusbacin A_3. Katanosin B and plusbacin A_3 also inhibited lipid intermediates formation, with IC_{50}s of 2.2 and 2.3 μg/ml, respectively (Fig. 2C), which were a few times higher than those for nascent peptidoglycan formation. On the other hand, vancomycin did not inhibit the formation of lipid intermediates even at the highest concentration tested (50 μg/ml), as shown in the literature (20). Excessive accumulation of lipid intermediates was observed at concentrations of vancomycin ranging from 6.25 to 25 μg/ml, which seemed to be caused by inhibition of the following reaction, transglycosylation, with little effect on lipid intermediate formation. These results indicated that katanosin B and plusbacin A_3 inhibited the steps preceding transglycosylation in the peptidoglycan synthesis pathway. Both katanosin B and plusbacin A_3 showed IC_{50} differences between lipid intermediates and nascent peptidoglycan formation, which indicated that both katanosin B and plusbacin A_3 also inhibited the transglycosylation step.

Suppression of inhibition by acetyl-Lys-D-Ala-D-Ala. Vancomycin inhibits transglycosylation via binding to the acyl-D-alanyl-D-Alanine (D-Ala-D-Ala) terminus of the lipid intermediates (1, 32). Acetyl-Lys-D-Ala-D-Ala, an analog of the terminus, suppressed the inhibition of transglycosylation by vancomycin effectively, but did not suppress those by katanosin B and plusbacin A_3 even at the highest concentration tested (800 μg/ml). MICs of katanosin B and plusbacin A_3 against *S. aureus* SRM133 were not affected by addition of 50 μg of acetyl-Lys-D-Ala-D-Ala per ml, while the MIC of vancomycin increased drastically, from 1.56 to 50 μg/ml.

Antagonism of antibacterial activity by a wall-membrane particulate. Inhibition zones surrounding disks containing katanosin B, plusbacin A_3, and vancomycin were distorted by the presence of a wall-membrane particulate (Fig. 5). On the other hand, inhibition zones made by methicillin, fosfomycin, and erythromycin were not affected (data not shown).

DISCUSSION

This study showed that both katanosin B and plusbacin A_3 can inhibit peptidoglycan synthesis. Their close MIC and IC_{50} values for [^{14}C]GlcNAc incorporation and nascent peptidoglycan formation suggested that inhibition of peptidoglycan synthesis leads to the antimicrobial activity. Katanosin B and plusbacin A_3 inhibited nascent peptidoglycan formation as well as vancomycin, whereas they also inhibited lipid intermediate formation, unlike vancomycin, although their IC_{50}s for lipid intermediate formation were a few times higher than those for nascent peptidoglycan formation. Acetyl-Lys-D-Ala-D-Ala neither suppressed the inhibition of nascent peptidoglycan formation by katanosin B and plusbacin A_3 nor reduced susceptibility to the two drugs, unlike its suppressive effects on vancomycin. These results indicated that katanosin B and plusbacin A_3 are inhibitors of peptidoglycan synthesis with a mode distinct from that of vancomycin. The different mode of action would mean that mechanisms of resistance to vancomycin would be ineffective against katanosin B and plusbacin A_3. In fact, the two drugs were active against vancomycin-resistant enterococci as well as against intermediate vancomycin-resistant *S. aureus*, whose resistance has been indicated to be due to increased vancomycin-binding ability (9, 10). The peptidoglycan synthesis pathway is an attractive target for antibacterial agents in terms of specificity for bacteria. Thus, katanosin B and plusbacin A_3 are potential candidates for the development of new therapeutic drugs.

The mode of action of katanosin B and plusbacin A_3 is not yet precisely understood. However, their inhibition seems to be the result of binding to lipid intermediates, substrates of several successive enzymes, rather than a direct effect on

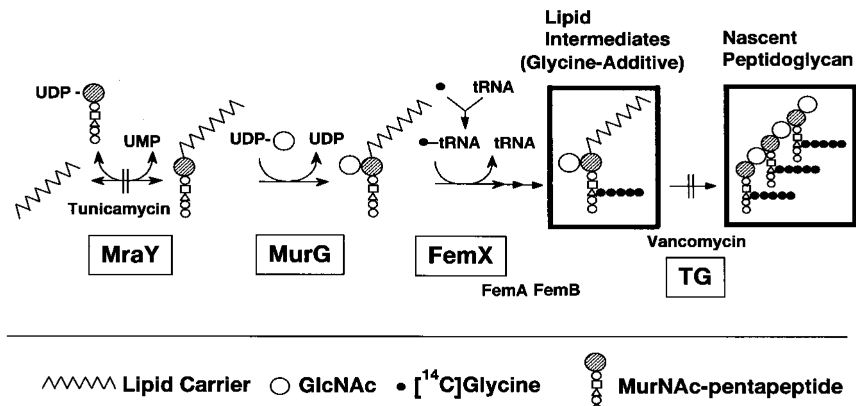

FIG. 3. Membrane pathways of peptidoglycan synthesis in *S. aureus*. [^{14}C]glycine is incorporated into lipid intermediates (glycine-additive) and nascent peptidoglycan, surrounded by bold rectangles. TG, transglycosylase. Enzymes essential for bacterial growth are boxed.

some enzyme, because both drugs inhibited at least two steps, the formation of lipid intermediates and transglycosylation. This speculation was supported by the antagonism of antibacterial activity by a wall-membrane particulate. The previous report showed that lysobactin, an analog of katanosin B, became bound to a cell wall preparation from *S. aureus* (2), and ramoplanin, an inhibitor of MurG, which is also a cyclic depsipeptide containing a lactone linkage, became bound to lipid intermediates (5, 40). The binding site of katanosin B and plusbacin A_3 should be other than acyl-D-Ala-D-Ala, thus differing from vancomycin.

The MIC of vancomycin was lower than expected from the IC_{50} for nascent peptidoglycan formation, which might be due to the additional effect of inhibition of transpeptidation, another target of vancomycin following transglycosylation (32).

Direct assay for in vitro transglycosylase activity entailed laborious work with *S. aureus* enzyme because of the difficulty of purifying both the substrate and enzyme. While high-molecular-weight penicillin-binding proteins (PBPs) of *Escherichia coli* proved to have transglycosylase activity (15, 16, 27), none of the PBPs of *S. aureus* had such activity, although gene analysis revealed that staphylococcal PBP2 included a transglycosylase domain (25, 30). The staphylococcal major transglycosylase gene remains to be identified. The preceding reactions were also too cumbersome to detect because of the laborious preparation of the substrates and enzymes, although recently, MraY and MurG activities are being assayed with *E. coli* enzymes prepared by gene cloning (4, 7, 8, 14, 22). The staphylococcal *murG* gene has yet to be identified, while the staphylococcal *mraY* gene has already been identified and cloned, showing it to have the same function as that of *E. coli* (3). The *fmhB* gene was recently identified as being essential and specific for staphylococcal cells (33, 42). We could detect both staphylococcal transglycosylation and its foregoing reactions without particular purification of enzymes and substrates by taking advantage of staphylococcus-specific incorporation

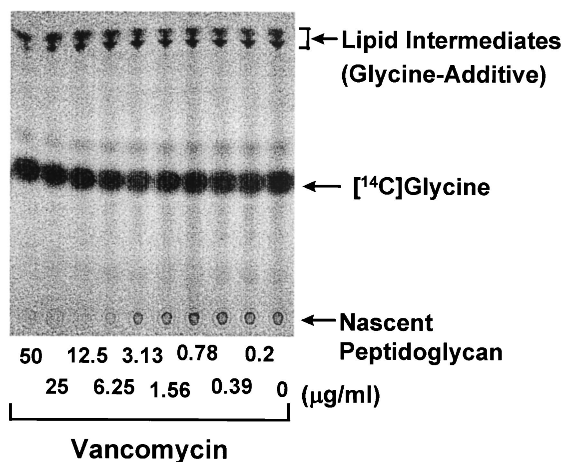

FIG. 4. Detection of inhibition of peptidoglycan synthesis. The reaction products were separated by TLC as described in Materials and Methods. A representative autoradiogram indicating the effect of vancomycin on the formation of lipid intermediates ($R_f \approx 0.9$) and nascent peptidoglycan (at the origin) is shown.

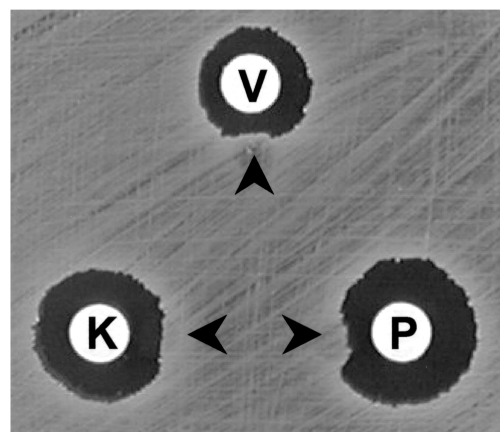

FIG. 5. Antagonism of antibacterial activity by a wall-membrane particulate. Wall membrane particulates were spotted at the positions shown by arrow heads. V, vancomycin; K, katanosin B; P, plusbacin A_3.

of glycine into peptidoglycan. Thus, this crude assay system may be useful for simple evaluation of inhibitors of staphylococcal peptidoglycan synthesis, especially MraY, MurG, FemX, and transglycosylase reactions, which are regarded as important targets for drug discovery.

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