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The Lantibiotic Mersacidin Inhibits Peptidoglycan Synthesis by Targeting Lipid II

HEIKE BRÖTZ,[1] GABRIELE BIERBAUM,[1] KLAUS LEOPOLD,[2] PETER E. REYNOLDS,[3] AND HANS-GEORG SAHL[1]*

Institut für Medizinische Mikrobiologie und Immunologie, Universität Bonn, D-53105 Bonn,[1] and Institut für Biochemie der Medizinischen Fakultät, Universität Erlangen, D-91054 Erlangen,[2] Germany, and Department of Biochemistry, University of Cambridge, Cambridge CB2 1QW, United Kingdom[3]

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The lantibiotic mersacidin exerts its bactericidal action by inhibition of peptidoglycan biosynthesis. It interferes with the membrane-associated transglycosylation reaction; during this step the ultimate monomeric peptidoglycan precursor, undecaprenyl-pyrophosphoryl-MurNAc-(pentapeptide)-GlcNAc (lipid II) is converted into polymeric nascent peptidoglycan. In the present study we demonstrate that the molecular basis of this inhibition is the interaction of mersacidin with lipid II. The adsorption of [^{14}C]mersacidin to growing cells, as well as to isolated membranes capable of in vitro peptidoglycan synthesis, was strictly dependent on the availability of lipid II, and antibiotic inhibitors of lipid II formation strongly interfered with this binding. Direct evidence for the interaction was provided by studies with isolated lipid II. [^{14}C]mersacidin associated tightly with [^{14}C]lipid II micelles; the complex was stable even in the presence of 1% sodium dodecyl sulfate. Furthermore, the addition of isolated lipid II to the culture broth efficiently antagonized the bactericidal activity of mersacidin. In contrast to the glycopeptide antibiotics, complex formation does not involve the C-terminal D-alanyl–D-alanine moiety of the lipid intermediate. Thus, the interaction of mersacidin with lipid II apparently occurs via a binding site which is not targeted by any antibiotic currently in use.

The family of lantibiotics comprises an increasing number of uniquely modified antibacterial peptides which are produced by a variety of gram-positive species (for a review, see reference 32). They are currently divided into two major groups (19, 32): the elongated, amphipathic, pore-forming type A lantibiotics, such as Pep5 or nisin (26, 31), and the globular peptides of the type B category, which appear to inhibit enzyme reactions (8, 15, 34). Mersacidin and actagardine (formerly "gardimycin"), another lantibiotic employed in this study, are representatives of the latter group. Both peptides contain four intramolecular thioether bridges, formed predominantly by β-methyllanthionine residues, which impose a globular shape and restricted flexibility on the molecules (10, 41). Furthermore, mersacidin and actagardine are of similar sizes (1,825 and 1,890 Da, respectively) and hydrophobicities and contain a conserved sequence motif which comprises one entire ring structure (8).

Previous studies on the mode of action indicated that, unlike type A lantibiotics, mersacidin did not impair the overall integrity of the cytoplasmic membrane (7); instead, it selectively blocked peptidoglycan metabolism and caused cell lysis in staphylococci (7, 25). Accumulation of the ultimate cytoplasmic peptidoglycan precursor, UDP-MurNAc-pentapeptide, in mersacidin-treated cells suggested blockage of a membrane-associated biosynthetic step, which was identified as the transglycosylation reaction by using a wall membrane preparation of *Bacillus megaterium* (8). Similar experiments were conducted with actagardine, and these indicated that its bactericidal activity is also based on inhibition of peptidoglycan synthesis at the level of transglycosylation (8, 34).

The aim of the present study was to investigate the molecular mechanism of this inhibition. Binding studies were conducted to determine whether mersacidin interferes with transglycosylation directly as a competitive enzyme inhibitor or whether it forms a complex with the peptidoglycan precursor and thus sterically prevents the action of transglycosylases.

* Corresponding author. Mailing address: Institut für Medizinische Mikrobiologie und Immunologie der Universität Bonn, Sigmund-Freud-Str. 25, D-53105 Bonn, Germany. Phone: (49)228/2875704. Fax: (49)228/2874808. E-mail: Sahl@mibi03.meb.uni-Bonn.de.

MATERIALS AND METHODS

Abbreviations. The following abbreviations are used in this article: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; GlcNAc, *N*-acetylglucosamine; HPLC, high-pressure liquid chromatography; MOPS, *N*-morpholinepropanesulfonic acid; MurNAc, *N*-acetylmuramic acid; PAGE, polyacrylamide gel electrophoresis, SDS, sodium dodecyl sulfate.

Bacterial strains. *Bacillus cereus* T (21) and *Bacillus* sp. strain HIL Y-85,54728 (10) were kindly provided by J.-V. Höltje (Tübingen, Germany) and Hoechst AG (Frankfurt, Germany), respectively. *Micrococcus luteus* ATCC 4698, *Staphylococcus simulans* 22 (4), and *B. megaterium* KM (28) were employed as indicator strains.

Chemicals and antibiotics. Commercially available compounds were obtained from the following manufacturers: UDP-[^{14}C]GlcNAc, Amersham-Buchler, Braunschweig, Germany; penicillin G, Hoechst; GlcNAc-β-1,4-MurNAc-Ala–D-iso-Gln and [^{14}C]glycine, ICN, Eschwege, Germany; vancomycin, Lilly, Giessen, Germany; CCCP, bisacetyl-Lys–D-Ala–D-Ala, dicalcium pyrophosphate, dimyristoylphosphatidylcholine, sodium deoxycholate, and UDP-GlcNAc, Sigma, Munich, Germany; bacitracin, Serva, Heidelberg, Germany. Mersacidin and moenomycin were kindly supplied by Hoechst, and ramoplanin and actagardine were kindly supplied by Merrel Dow/Lepetit (Gerenzano, Italy). Crude actagardine (85% pure) was further purified on a Poros 10 R2 reversed-phase HPLC column as described previously for mersacidin (5). UDP-MurNAc-pentapeptide was isolated from the cytoplasm of vancomycin-treated cells as reported previously (8). *S. simulans* 22 or *B. cereus* T was used for the purification of the lysine- or diaminopimelic acid-containing compound, respectively.

Synthesis and purification of [^{14}C]mersacidin. [^{14}C]mersacidin was prepared by in vivo labeling. *Bacillus* sp. strain HIL Y-85,54728 was grown in 200 ml of a synthetic medium, as reported previously (5). Fourteen and a half hours after inoculation, 1 mCi of [^{14}C]glycine (63 mCi/mmol) was added. After a further 65 h, the supernatant was applied to a column of the polystyrene resin Serdolit AD-2 (2.2 by 10.5 cm) (Serva), which was eluted in batch type chromatography successively with 240 ml of water, 240 ml of 50% methanol in 50 mM potassium phosphate buffer (pH 7), and a stepwise gradient from this solvent to 90% 2-propanol in 0.2% trifluoroacetic acid (pH 2.2). Elution steps of 5, 7.5, 10, 20, 30, 40, 50, 60, 70, and 90% of the second eluent were used at a volume of 40 ml each. The 40% fraction, containing most of the mersacidin, was recovered, and methanol was evaporated in a desiccator. Further purification was achieved by

perfusion chromatography on a Poros 10 R2 column (4.6 by 100 mm, Perseptive Biosystems, Freiburg, Germany) as described previously (5). Four milligrams of [^{14}C]mersacidin with a specific activity of 1.7 mCi/mmol, corresponding to a yield of 75%, was obtained.

Synthesis and purification of [^{14}C]lipid II. [^{14}C]lipid II was synthesized in vitro by protoplasts of *M. luteus* from soluble UDP-linked peptidoglycan precursors. A culture of *M. luteus* ATCC 4698 was grown in tryptone soy broth to an A_{600} of 1, harvested rapidly (10,000 × *g*, 7 min, 2°C), washed with 50 mM Tris HCl (pH 7.5) at 4°C, and resuspended in 10 ml of the same buffer containing 10 mM $MgCl_2$. Protoplasts from 2 liters of culture, prepared by lysozyme digestion according to the method of Katz et al. (20), were gently stirred for in vitro synthesis of [^{14}C]lipid II (labeled in the GlcNAc moiety), with UDP-MurNAc-pentapeptide (0.07 mM; lysine containing) and UDP-[^{14}C]GlcNAc (0.07 mM; 2.5 mCi/mmol) in 6 ml of 50 mM Tris HCl (pH 8)–10 mM $MgCl_2$–3.4 mM sodium deoxycholate for 2 h at 20°C. The membrane lipids were extracted with *n*-butanol–pyridinium acetate. The final *n*-butanol phase (20 ml) was applied directly to a column of DEAE-cellulose (0.9 by 30 cm) (Serva) at a flow rate of 0.2 ml/min; the DEAE-cellulose had previously been transferred into the acetate form (12). The column was developed at a flow rate of 0.4 ml/min with 120 ml of 99% methanol followed by a gradient of ammonium acetate in 99% methanol (modification of the method of van Heijenoort et al. [40]): 100 min isocratic at 0.1 M ammonium acetate, 0.1 to 0.4 M in 60 min, 45 min isocratic at 0.4 M, 0.4 to 2 M in 350 min; ammonium acetate in 99% methanol was prepared as described by Dankert et al. (12). [^{14}C]lipid II was eluted between 0.2 and 0.8 M ammonium acetate. Radioactive fractions were pooled (21 ml), diluted with chloroform to a chloroform/methanol ratio of 5:1 (vol/vol), and directly applied, at a flow rate of 0.4 ml/min, to a silicic acid column (0.9 by 23 cm) (ICN) equilibrated with the same solvent mixture. The column was developed at a flow rate of 0.8 ml/min with 20 ml of chloroform-methanol (5:1) and a linear gradient towards 100% methanol (gradient volume, 150 ml). [^{14}C]lipid II was eluted at a solvent composition of 50% methanol. Radioactive fractions were pooled, evaporated to dryness at 3°C in a rotary evaporator, redissolved in 5 ml of chloroform-methanol (1:1), and stored at −20°C. The yield was 70 nmol of [^{14}C]lipid II as determined by [^{14}C]GlcNAc content. The phosphorus content (determined as described by Chen et al. [11]) was 5 mol per mol of disaccharide-pentapeptide, indicative of the presence of residual unlabeled phospholipids (1, 20). [^{14}C]lipid II was the only labeled compound in the preparation and had a specific activity of 2.5 mCi/mmol.

Binding of [^{14}C]mersacidin to intact cells. Binding studies were conducted with exponentially growing cells. Either *M. luteus* ATCC 4698 or *S. simulans* 22 was grown in half-concentrated Mueller-Hinton broth at 33 or 37°C, respectively. At an A_{600} of 0.4, [^{14}C]mersacidin was added. For all binding studies with whole cells, concentrations of 11 μg/ml (6 μM, corresponding to the MIC) were used for *S. simulans* and, unless otherwise indicated, 1 μg/ml (0.55 μM, corresponding to 10 times the MIC) was used for *M. luteus*. The amount of mersacidin bound to the cells was determined by filtering culture aliquots (2.5 ml) on hydrophilic Durapore filters (Millipore, Eschborn, Germany). The dried filters were counted in Quickzint 100 (Zinsser, Frankfurt, Germany) in a 1900 CA Tri-Carb liquid scintillation counter (Packard, Zurich, Switzerland).

To determine the mersacidin-binding capacity of de-energized cells and the effect of vancomycin on binding, a culture of *S. simulans* 22 was grown to an A_{600} of 0.4 and divided into three aliquots. One aliquot was incubated with vancomycin (5.4 μM, 20 times the MIC) for 5 min. Then, both the vancomycin-treated aliquot and a second, untreated aliquot were de-energized by the addition of CCCP (100 μM), while a third aliquot served as a control. After 30 min of de-energization, [^{14}C]mersacidin was added to each aliquot, and incubation was continued for 5 min before the amount of adsorbed mersacidin was determined.

The binding capacity of *M. luteus* ATCC 4698 for [^{14}C]mersacidin after preincubation with other inhibitors of peptidoglycan biosynthesis was investigated by pretreating growing cells with various antibiotics at 33°C for 5 min; subsequently, [^{14}C]mersacidin was added, and after 5 min the amount of adsorbed lantibiotic was determined. The following antibiotic concentrations were used: mersacidin (unlabeled), 1.1 and 5.5 μM; actagardine, 7.4 μM; vancomycin, 1.3 and 5.5 μM; moenomycin, 63 μM; penicillin G, 11.9 μM; bacitracin, 2.1 μM; ramoplanin, 0.16 and 5.5 μM.

Binding studies with starved cells were carried out by growing *M. luteus* ATCC 4698 to an A_{600} of 0.4, washing the cells twice with 50 mM sodium phosphate buffer (pH 7), and incubating the culture for a further 2 h in the same buffer, prior to the addition of [^{14}C]mersacidin.

Binding of [^{14}C]mersacidin to isolated membranes. Binding studies were performed with either a wall membrane or a protoplast membrane fraction of *B. megaterium* KM. The wall membrane preparation was obtained by mechanical disruption of whole cells and differential centrifugation as described previously (28). The protoplast membranes were prepared from nonreconditioned protoplasts as described by Reynolds (29). For mersacidin-binding experiments, either the wall membrane preparation (60 μg of protein) or the protoplast membranes (150 μg of protein) were incubated with UDP-MurNAc-pentapeptide (0.4 mM; containing diaminopimelic acid), UDP-GlcNAc (0.4 mM), and [^{14}C]mersacidin (100 μg/ml) in a total volume of 30 μl of 50 mM Tris HCl (pH 7.8)–10 mM $MgCl_2$ for 45 min at 23°C. Unbound mersacidin was removed by washing the membranes twice with 1.4 ml of the same buffer (8,000 × *g*, 10 min), prior to liquid scintillation counting. The effect of actagardine, vancomycin, moenomycin, bacitracin, or ramoplanin was tested by simultaneously adding the antibiotic (300 μg/ml) and [^{14}C]mersacidin to the incubation mixture. To investigate the binding of [^{14}C]mersacidin to membranes in the absence of soluble peptidoglycan precursors, UDP-MurNAc-pentapeptide and UDP-GlcNAc were omitted from the incubation mixture. In vitro synthesis of peptidoglycan and lipid II was monitored by incubating the membrane preparations (100 μg of protein) with UDP-MurNAc-pentapeptide (0.4 mM) and UDP-[^{14}C]GlcNAc (0.4 mM, 1.3 mCi/mmol) in 30 μl of the same buffer. Samples were then separated by paper chromatography and analyzed by liquid scintillation counting (28).

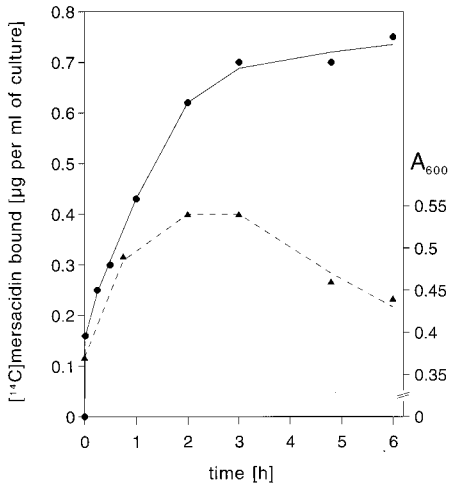

FIG. 1. Binding of [^{14}C]mersacidin to *M. luteus* ATCC 4698. At time zero an exponentially growing culture was treated with [^{14}C]mersacidin (7 μg/ml; 70 times the MIC), and binding (●) was determined by filtration of culture aliquots. ▲, A_{600}.

Binding studies with isolated cell walls or phospholipid liposomes. Five hundred micrograms of lyophilized cell walls, purified from *M. luteus* ATCC 4698 by tryptic digestion and SDS extraction (7), was incubated for 30 min with 1 μg of [^{14}C]mersacidin in 1 ml of 50 mM Tris HCl (pH 7). Liposomes from 2 mg of dimyristoylphosphatidylcholine, prepared in 50 mM MOPS (pH 7)–50 mM KCl by three freeze-thaw cycles as described by Davidson et al. (13), were incubated with 3 μg of [^{14}C]mersacidin for 30 min in 1 ml of the same buffer. The amount of mersacidin bound to the cell walls or liposomes was determined by filtration on Durapore filters, as described above.

Gel electrophoresis. Various concentrations of [^{14}C]mersacidin (in 1 μl of methanol) and [^{14}C]lipid II (in 30 to 60 μl of chloroform-methanol [1:1]) were mixed, and the solvents were evaporated in a desiccator. The samples were incubated in 10 μl of sample buffer (63 mM Tris HCl [pH 6.8], 10% glycerol, 0.025% bromphenol blue) for 15 min at 20°C and were subjected to nondenaturing PAGE (stacking gel, 4% polyacrylamide [pH 7]; separating gel, 20% polyacrylamide [pH 8.3]). The gels were dried and exposed to an X-ray film for 2 months at −70°C. A second set of samples was analyzed with 1% SDS in the sample buffer, 0.1% SDS in the running buffer, but no SDS in the gels.

The binding of [^{14}C]mersacidin to *M. luteus* ATCC 4698 was analyzed by conventional SDS-PAGE as described by Laemmli (23) in the presence of 2% SDS in the sample buffer and 0.1% SDS in both the running buffer and the gels. The cells were grown for 1 h in the presence of [^{14}C]mersacidin, washed to remove the unbound lantibiotic, and boiled for 15 min in sample buffer containing 2% SDS and 5% 2-mercaptoethanol. Cell debris was removed by centrifugation, and compounds in the supernatant were separated on 20% gels.

MIC determinations. MICs were determined for *M. luteus* ATCC 4698 by broth microdilution, as reported previously (7). Antagonizing agents were diluted together with the antibiotics, thus keeping a constant molar ratio (see Table 2). Oligomeric cell wall fragments of *M. luteus* ATCC 4698 were obtained by digesting 7 mg of purified cell walls with 100 μg of lysozyme (48,000 U/mg) in 0.5 ml of 20 mM potassium phosphate buffer (pH 6.5) for 18 h at 37°C. After 1 h of boiling and subsequent centrifugation (12,000 × *g*, 10 min), the supernatant containing cell wall subunits was lyophilized and used in MIC determinations.

RESULTS

Binding studies with growing cells. Addition of [^{14}C]mersacidin to *M. luteus* ATCC 4698 resulted in immediate binding of 22% of the total amount adsorbed in the course of the experiment (Fig. 1). Subsequent adsorption continued in an approximately linear fashion for about 2 h, while cell growth pro-

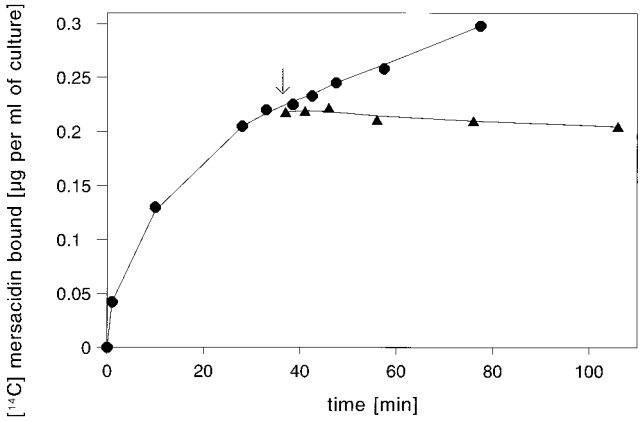

FIG. 2. Chase experiment with [^{14}C]mersacidin. *M. luteus* ATCC 4698 was grown in the presence of [^{14}C]mersacidin (1 μg/ml). At the time indicated by the arrow, the culture was divided into two aliquots, one of which was chased with a 900-fold excess of unlabeled mersacidin (▲), while the other served as a control (●).

ceeded, as measured by turbidity at 600 nm. As was previously observed for *S. simulans* 22 (7, 25), treatment of *M. luteus* with mersacidin did not result in immediate cell lysis. Instead, cell density increased over a period corresponding to approximately one generation time, followed by a slow reduction in optical density. Cell lysis did not cause a release of [^{14}C]mersacidin from its target sites. Furthermore, the binding of mersacidin was sufficiently strong to withstand washing of the cells with buffer or methanol on filters in the course of the binding assay. This result is in accordance with the observation that [^{14}C]mersacidin, once adsorbed to the cells, was not displaced by the addition of a 900-fold excess of unlabeled mersacidin (Fig. 2).

Approximately 2×10^5 binding sites per cell were found for growing cells of *M. luteus* ATCC 4698, and 7×10^4 binding sites per cell were found for *S. simulans* 22. Similar numbers were reported for bacitracin (2×10^5 molecules per cell of *M. luteus* [38]) and ramoplanin (5×10^4 molecules per cell of *Staphylococcus aureus* [35]). Bacitracin forms a complex with undecaprenylpyrophosphate (36), while for ramoplanin an interaction with the peptidoglycan intermediate lipid I has been discussed (35). In contrast, the reported numbers for transglycosylases are in the order of 10^3 molecules per cell (14, 16). This suggests that the demonstrated effect of mersacidin on transglycosylation is rather based on the interaction with the substrate lipid II than with the enzymes.

Effect of de-energization on the binding of [^{14}C]mersacidin. De-energized cells had a strongly reduced binding capacity for [^{14}C]mersacidin. When *M. luteus* ATCC 4698 was starved in buffer for 2 h prior to the addition of the label, the amount of mersacidin bound was up to 30 times lower than that adsorbed by an exponentially growing culture. Similarly, treatment of *S. simulans* 22 for 30 min with the protonophore CCCP reduced the amount of [^{14}C]mersacidin adsorbed to 14% of that of an untreated control culture. It is conceivable that during de-energization the available lipid II molecules are converted into polymeric peptidoglycan, while their energy-requiring de novo synthesis is prevented under these circumstances. Therefore, we tried to trap lipid II in the monomeric state by vancomycin before de-energizing the cells. To this end, we incubated an additional culture aliquot of *S. simulans* with vancomycin for 5 min prior to the addition of CCCP, which increased the binding capacity of de-energized cells from 14 to 84%.

TABLE 1. Binding of [^{14}C]mersacidin to *M. luteus* ATCC 4698 after preincubation[a] with inhibitors of cell wall biosynthesis

Antibiotic	Concn[b]	Antibiotic/ [^{14}C]mersacidin[c]	Binding (%)[d]
Mersacidin			
Unlabeled	20	2	17
Unlabeled	100	10	7
Actagardine	20	13.5	4
Vancomycin	20	2.4	98
	83	10	85
Moenomycin	20	115	125
Penicillin G	20	21.6	100
Ramoplanin	20	0.3	20
	667	10	2
Bacitracin	20	3.8	6

[a] 5 min.
[b] Multiple of the MIC.
[c] Molar ratio.
[d] Relative to the control culture without preincubation.

Effects of peptidoglycan biosynthesis inhibitors on the binding of [^{14}C]mersacidin. We selected several antibiotics known to interfere with various membrane-associated steps in peptidoglycan synthesis (Fig. 3) and determined their influence on the binding of [^{14}C]mersacidin to a growing culture of *M. luteus* ATCC 4698 (Table 1). Binding assays were conducted by preincubating the cells with the respective antibiotics (20 times the MIC) for 5 min prior to the addition of labeled mersacidin. When the antibiotic concentration of 20 times the MIC corresponded to a smaller or only slightly higher molarity compared

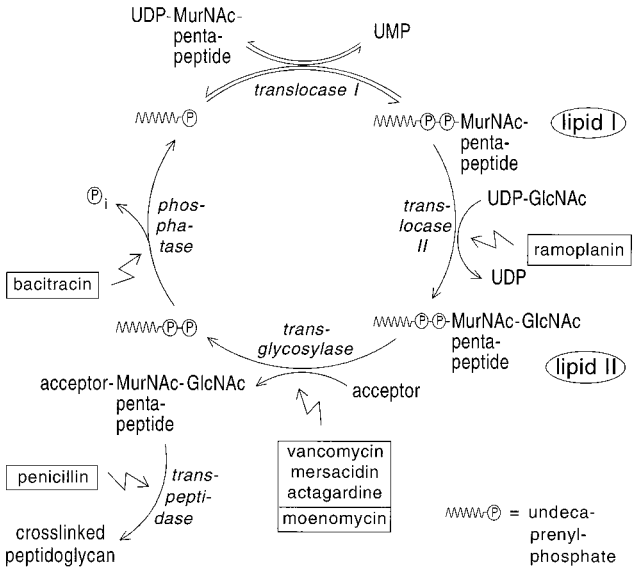

FIG. 3. Cycle of the lipid carrier in peptidoglycan biosynthesis. The target reactions of the antibiotics employed in this study, all of which interfere with one of the membrane-associated stages, are depicted. Moenomycin and penicillin interact directly with the respective enzymes (6, 39), while for vancomycin and bacitracin, complex formation with the peptidoglycan precursors has been established (27, 37). For ramoplanin an interaction with lipid I has been discussed (35).

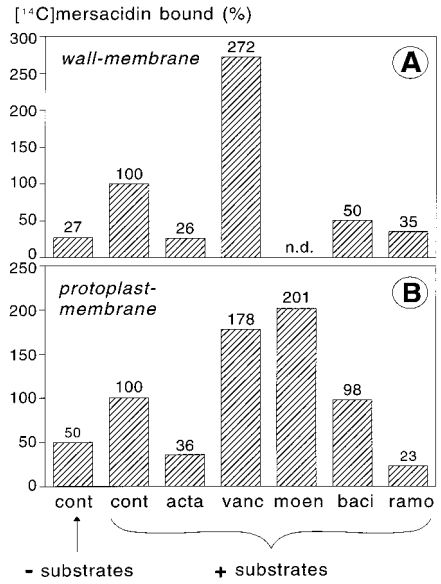

FIG. 4. Binding of [^{14}C]mersacidin to isolated membranes of *B. megaterium* KM. Adsorption to either a wall fragment-containing membrane preparation capable of synthesizing polymeric peptidoglycan (A) or a protoplast membrane preparation which forms lipid II but no peptidoglycan (B) was measured. The binding capacity of membranes performing peptidoglycan synthesis in vitro in the presence of soluble UDP-linked peptidoglycan precursors (+ substrates) was compared to that of membranes in the absence of these precursors (− substrates). The effects of an incubation of the membranes with the combination of [^{14}C]mersacidin (100 μg/ml) and 300 μg/ml of either actagardine (acta), vancomycin (vanc), moenomycin (moen), bacitracin (baci), or ramoplanin (ramo) are shown. The amount of mersacidin bound is given as a percentage of the binding capacity of control membranes (cont) in the presence of substrates. The 100% value corresponds to an adsorption of 3.8 ng of [^{14}C]mersacidin per μg of membrane protein for the wall membrane preparation and 4.7 ng/μg of protein for the protoplast membrane preparation. n.d., not determined.

to [^{14}C]mersacidin, the experiment was performed additionally at a molar ratio of antibiotic to labeled mersacidin of 10 to 1. Pretreatment with unlabeled mersacidin strongly reduced the binding capacity for [^{14}C]mersacidin, as did preincubation with the structurally related lantibiotic actagardine (Table 1). Binding was also reduced by ramoplanin and bacitracin, both of which interfere with the formation of lipid II (Fig. 3). In contrast, the competitive enzyme inhibitor of transglycosylases or transpeptidases—moenomycin or penicillin G, respectively (6, 39)—did not impede binding, indicating that mersacidin is unlikely to interact directly with one of these enzymes. Although the binding of [^{14}C]mersacidin was apparently dependent on the availability of lipid II in the membrane, the presence of vancomycin, which binds to lipid II itself (for a review, see reference 30), did not markedly reduce adsorption. Similar results were obtained when the binding assay was performed with *S. simulans* 22 as an indicator organism.

Binding studies with isolated cell fractions. The binding of [^{14}C]mersacidin to isolated membranes was determined with two different membrane preparations of *B. megaterium* KM under conditions which enabled them to synthesize lipid II or peptidoglycan in vitro (Fig. 4). The binding capacity of a wall fragment-containing membrane preparation, capable of synthesizing polymeric peptidoglycan from the soluble peptidoglycan precursors UDP-MurNAc-pentapeptide and UDP-GlcNAc (28), was compared with that of a preparation obtained by lysis of nonreconditioned protoplasts (29). The protoplast membranes retained the ability to synthesize lipid II, although the amount of peptidoglycan formed was only 2.5% of that synthesized by the wall membrane fraction. Both membrane preparations adsorbed significantly more [^{14}C]mersacidin in the presence than in the absence of the UDP-linked precursors (Fig. 4). The increase in the number of target sites is consistent with the de novo synthesis of lipid II in vitro, whereas biosynthesis of transglycosylase molecules is not possible under these conditions. The effects of several inhibitors of peptidoglycan synthesis on the binding capacity of the membranes (Fig. 4) support the results obtained with growing cells. The adsorption of [^{14}C]mersacidin was effectively prevented by actagardine and ramoplanin. Bacitracin interfered with the binding to the wall membrane but not to the protoplast membrane fraction, because the target of bacitracin, undecaprenylpyrophosphate, is released after transglycosylation (Fig. 3) and is thus not present in the protoplast membrane preparation. In contrast, the inhibitors of transglycosylation, moenomycin and vancomycin, which induce an accumulation of lipid II in the membranes (8, 28, 29, 35) significantly increased their binding capacity.

Binding studies with protoplast membranes of *M. luteus* ATCC 4698 led to similar findings. When filtration assays were performed to determine the affinity of [^{14}C]mersacidin for phosphatidylcholine liposomes or purified peptidoglycan sacculi of *M. luteus* ATCC 4698, only background levels of radioactivity were detectable on the filters.

Interaction of mersacidin with purified lipid II. Various amounts of purified [^{14}C]lipid II and either [^{14}C]mersacidin or unlabeled mersacidin were mixed in a small volume of chloroform-methanol (1:1). Following evaporation of the solvents, the samples were suspended in buffer and analyzed by nondenaturing PAGE (Fig. 5). Under nondenaturing conditions (Fig. 5A), [^{14}C]lipid II was retained at the upper edge of the separating gel, probably due to the formation of micelles, while [^{14}C]mersacidin migrated further into the gel. The passage of [^{14}C]mersacidin into the gel was completely prevented after incubation with equimolar or higher amounts of [^{14}C]lipid II, indicative of an interaction of the lantibiotic with the lipid II micelles; only when the amount of [^{14}C]mersacidin exceeded that of [^{14}C]lipid II did the surplus mersacidin move into the gel. The presence of a high concentration of mersacidin apparently influenced the size or surface properties of the lipid II micelles. When mixed with a ninefold molar excess of (in this case unlabeled) mersacidin, less [^{14}C]lipid II was visible at the edge of the separating gel (Fig. 5A, lane 4); it reappeared when the sample and running buffer were supplied with SDS (1 and 0.1%, respectively; Fig. 5B, lane 4), suggesting that it had already been prevented from entering the stacking gel. In addition, the interaction with mersacidin markedly increased the stability of the lipid II micelles, since they withstood solubilization by SDS in the presence of, but not in the absence of, mersacidin (Fig. 5B).

In order to investigate whether the interaction of mersacidin with lipid II involves covalent binding, cells of *M. luteus* ATCC 4698, to which [^{14}C]mersacidin had been adsorbed, were boiled in the presence of 2% SDS. When SDS-PAGE was performed, only unbound mersacidin was detected in the gels (Fig. 5C).

Antagonization of the activity of mersacidin by purified lipid II. MIC determinations were conducted in order to examine whether the addition of purified lipid II to the culture broth is able to antagonize the activity of mersacidin (Table 2). Whereas the MIC of mersacidin for *M. luteus* ATCC 4698 was 0.1 μg/ml in the absence of extracellular lipid II, unhindered growth was still recorded at the highest concentration tested (2.5 μg/ml) in the presence of the lipid intermediate. A fourfold molar excess of lipid II over mersacidin was sufficient for

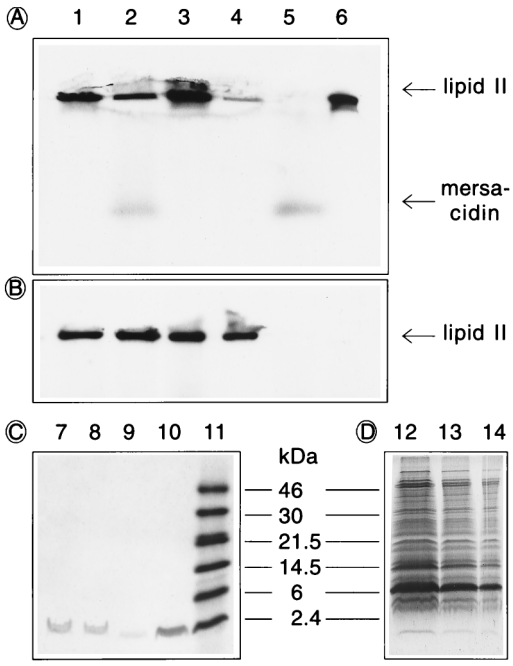

FIG. 5. (A and B) Interaction of mersacidin with isolated lipid II. Various amounts of [^{14}C]lipid II and either [^{14}C]mersacidin or unlabeled mersacidin were incubated and subjected to PAGE under nondenaturing conditions (A) or in the presence of 1% SDS in the sample buffer and 0.1% SDS in the running buffer (B). Lane 1, 0.4 nmol of [^{14}C]mersacidin and 0.4 nmol of [^{14}C]lipid II; lane 2, 0.8 nmol of [^{14}C]mersacidin and 0.4 nmol of [^{14}C]lipid II; lane 3, 0.4 nmol of [^{14}C]mersacidin and 0.8 nmol of [^{14}C]lipid II; lane 4, 4 nmol of unlabeled mersacidin and 0.4 nmol of [^{14}C]lipid II; lane 5, 0.4 nmol of [^{14}C]mersacidin; lane 6, 0.4 nmol of [^{14}C]lipid II. The lipid II bands mark the upper boundary of the separating gel. Most of the stacking gel was removed prior to autoradiography. (C and D) SDS-PAGE of *M. luteus* ATCC 4698 after adsorption of [^{14}C]mersacidin to the cells. (C) Autoradiogram; (D) Coomassie stain of gels run in parallel. Lanes 7 and 12, 5 mg of cells (wet weight); lanes 8 and 13, 2.5 mg of cells; lanes 9 and 14, 1 mg of cells; lane 10, 0.25 nmol of [^{14}C]mersacidin; lane 11, ^{14}C-methylated Rainbow molecular mass marker (Amersham-Buchler).

TABLE 2. Antagonization of mersacidin by isolated lipid II

Antagonist	Antagonist/antibiotic ratio[a]	MIC (μg/ml) for *M. luteus* ATCC 4698			
		Mersacidin	Vancomycin	Actagardine	Bacitracin
None		0.1	0.1	0.8	0.08
Lipid II	5	>2.5[b]	>2.5	ND[c]	0.08
UDP-MurNAc-pentapeptide	100	0.1	>10	ND	ND
UDP-MurNAc-pentapeptide and GlcNAc	100	0.1	ND	ND	ND
Moenomycin[d]	17	0.05	ND	ND	ND
Oligomeric *M. luteus* peptidoglycan[e]		0.2	>16	ND	ND
GlcNAc-MurNAc-Ala–D-iso-Gln	100	0.1	ND	ND	ND
	1,000	0.15			
GlcNAc-MurNAc-Ala–D-iso-Gln and PP_i[f]	100	0.1	ND	ND	ND
	1,000	0.15			
GlcNAc and UDP-GlcNAc	100	0.1	ND	ND	ND
Bisacetyl-Lys–D-Ala–D-Ala	10	0.1	0.2	0.8	ND
	100	0.1	>20	0.8	

[a] Antagonists were diluted along with the antibiotics, and throughout the dilution series the indicated molar ratio was kept constant.
[b] >, complete antagonization at the highest concentration tested.
[c] ND, not determined.
[d] A subinhibitory concentration of 1.5 μg/ml, corresponding to 40% of the MIC, was used.
[e] 2.3 mg of lysozyme-digested cell walls was mixed with 4 μg of the antibiotic and diluted concomitantly.
[f] The pyrophosphate (PP_i) concentration was kept constant at 0.5 mM throughout the dilution series.

this effect, indicating that the extracellular lipid II efficiently competed with the cell-bound target for the available mersacidin. None of the other compounds listed in Table 2, which represent or mimic parts of the lipid II molecule (Fig. 6), displayed the antagonizing effect, even at much higher concentrations. In contrast, vancomycin was antagonized by acyl-D-Ala–D-Ala-containing structures and by cell walls of *M. luteus*, where it most probably binds additionally to the acyl-Ala–D-Glu–Gly portion of the peptide side chain (17). As expected, the activity of bacitracin was not affected by lipid II in the supernatant, since its binding site is specific for undecaprenylpyrophosphate and the presence of a carbohydrate moiety on the lipid pyrophosphate prevents interaction (36).

DISCUSSION

The lantibiotic mersacidin inhibits peptidoglycan biosynthesis at the level of transglycosylation (8). The results presented here demonstrate that the molecular basis of this inhibition is the tight interaction with the membrane-bound peptidoglycan precursor, undecaprenyl-pyrophosphoryl-MurNAc-(pentapeptide)-GlcNAc (lipid II). (i) The numbers of binding sites determined by adsorption of [^{14}C]mersacidin are in agreement with a specific interaction of an antibiotic with a lipid intermediate in the peptidoglycan biosynthetic cycle. (ii) The binding capacities of *M. luteus* and *S. simulans* were influenced by the energy state of the cells, and the numbers of target sites were considerably reduced by de-energization with the protonophore CCCP or by starvation in buffer. (iii) The adsorption of [^{14}C]mersacidin to growing cells (Table 1), as well as to isolated membranes (Fig. 4), was strictly dependent on the availability of lipid II, and inhibitors of lipid II synthesis interfered with binding. (iv) Mersacidin strongly bound to purified micelles of lipid II during gel electrophoresis (Fig. 5), and lipid II completely antagonized the bacteriocidal activity of the lantibiotic (Table 2).

Although complex formation does not involve covalent

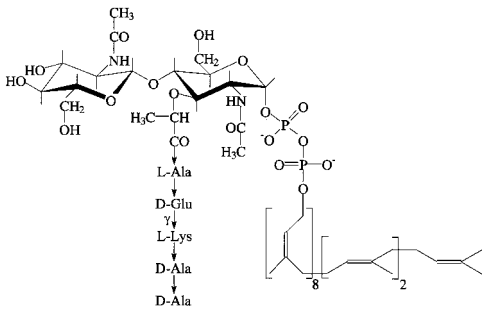

FIG. 6. Structure of the peptidoglycan precursor lipid II as synthesized in vitro by *M. luteus* membranes.

bonds (Fig. 5C), the association is rather tight since the complex did not dissociate in 1% SDS or upon washing of *M. luteus* cells with buffer, methanol, or a large excess of unlabeled mersacidin. Apparently, adsorption of mersacidin to lipid II is very specific and does not occur with either phospholipid liposomes or purified cell walls of *M. luteus*. The latter was reported for vancomycin (33), which also binds in large amounts to peptidoglycan sacculi of *Bacillus subtilis* (3). Furthermore, the number of binding sites is well in the range of the overall amount of lipid-bound cell wall intermediates; blocking of lipid II synthesis eliminated binding (Table 1).

The interaction of mersacidin with lipid II seems to involve substantial portions of both molecules. Even when employed at high concentrations, none of the individual building blocks of the lipid II molecule (Fig. 6) or structurally related compounds were able to antagonize the activity of the lantibiotic (Table 2). This provides some information on the molecular nature of the target site. Several lines of evidence indicate that mersacidin does not interact with the C-terminal D-alanyl–D-alanine portion of the lipid intermediate. (i) The lantibiotic is not antagonized by any of the acyl-D-Ala–D-Ala-containing structures listed in Table 2, including UDP-MurNAc-pentapeptide which contains the entire pentapeptide side chain. Therefore, its molecular mechanism of action differs from that of the glycopeptide antibiotic vancomycin, for which complex formation with this portion of the peptidoglycan precursors has been established (27; for reviews, see references 17 and 30). (ii) The binding of mersacidin to growing cells (Table 1), as well as to isolated membranes (Fig. 4), was not inhibited by vancomycin, indicating simultaneous adsorption and thus different binding sites for the two antibiotics. (iii) It has been shown previously that mersacidin is active against vancomycin-resistant *Enterococcus faecium*, which synthesizes an alternative peptidoglycan precursor terminating in D-alanyl–D-lactate, for which vancomycin has a low affinity, and that it inhibits in vitro peptidoglycan synthesis from UDP-MurNAc-tripeptide, a precursor which lacks the two C-terminal amino acid residues (8). Consequently, the peptide side chain of the lipid intermediate is unlikely to be involved in complex formation, leaving the disaccharide moiety, the pyrophosphate group, and the undecaprenyl residue as possible candidates for an interaction (Fig. 6). An involvement of the disaccharide headgroup is supported by the finding that even high concentrations of mersacidin did not interfere with the translocase II reaction (8); thus, mersacidin seems to have a significantly higher affinity for lipid II than for lipid I, which lacks the GlcNAc residue. On the other hand, the interaction of mersacidin with lipid II appears to involve more than just the disaccharide unit, as its affinity for lysozyme-digested cell walls of *M. luteus*, in which free disaccharide headgroups are available, as well as for GlcNAc-MurNAc-Ala–D-iso-Gln was too low to antagonize its growth-inhibitory activity (Table 2). Barrett et al. (2) observed an increased bactericidal activity of mersacidin in a calcium-enriched medium, which may hint at an involvement of the pyrophosphate moiety of the lipid intermediate. With respect to the possible ratio of mersacidin and lipid II in complex formation, it is noteworthy that lipid II micelles adsorbed approximately equimolar amounts of mersacidin (Fig. 5).

Of all inhibitors of transglycosylation that were employed in this study, only the structurally related lantibiotic actagardine interfered with the adsorption of mersacidin (Table 1; Fig. 4). The concentrations of actagardine necessary for inhibition of transglycosylation in vitro paralleled those of mersacidin and vancomycin but were 2 to 3 orders of magnitude higher than that of the competitive enzyme inhibitor moenomycin (8, 34, 35). This result suggests an interaction of actagardine with lipid II rather than with the transglycosylase and, together with the observations that it prevented the binding of mersacidin and that its activity was not antagonized by bisacetyl-Lys–D-Ala–D-Ala (Table 2), indicates that actagardine competes with mersacidin for the same target binding site. The two lantibiotics contain one ring structure that has been almost completely conserved in both molecules (8, 10, 41). It is tempting to suggest that this conserved sequence motif is the structural basis for their activity. Both peptides interact with a novel target site on the lipid II molecule and may therefore be the prototypes for a new class of chemotherapeutic agents. In this context it is noteworthy that both lantibiotics are also active against the pseudomurein-containing *Methanobacterium* archaebacteria (18, 22), suggesting that they bind to a highly conserved portion of the lipid intermediate. The promising in vivo activities of mersacidin against methicillin-resistant *Staphylococcus aureus* (9) and of actagardine against *Streptococcus pneumoniae* (24) indicate the potential of these lantibiotics for future development of drugs against these problematic pathogens, particularly since altered peptides can be constructed by manipulation of their structural genes (32).

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