

Isolation and Characterization of Genetically Engineered Gallidermin and Epidermin Analogs

BIRGIT OTTENWÄLDER,¹ THOMAS KUPKE,¹ SUSAN BRECHT,¹ VOLKER GNAU,² JÖRG METZGER,²
GÜNTHER JUNG,² AND FRIEDRICH GÖTZ^{1*}

Mikrobielle Genetik¹ and Institut für Organische Chemie,² Universität Tübingen, 72076 Tübingen, Germany

Received 4 May 1995/Accepted 15 August 1995

Gallidermin (Gdm) and epidermin (Epi) are highly homologous tetracyclic polypeptide antibiotics that are ribosomally synthesized by a *Staphylococcus gallinarum* strain and a *Staphylococcus epidermidis* strain, respectively. These antibiotics are secreted into media and are distinguished by the presence of the unusual amino acids lanthionine, 3-methylanthionine, didehydrobutyrine, and *S*-(2-aminovinyl)-*D*-cysteine, which are formed by posttranslational modification. To study the substrate specificities of the modifying enzymes and to obtain variants that exhibit altered or new biological activities, we changed certain amino acids by performing site-specific mutagenesis with the Gdm and Epi structural genes (*gdmA* and *epiA*, respectively). *S. epidermidis* Tü3298/EMS6, an *epiA* mutant of the Epi-producing strain, was used as the expression host. This mutant synthesized Epi, Gdm, or analogs of these antibiotics when the appropriate genes were introduced on a plasmid. No Epi or Gdm analogs were isolated from the supernatant when (i) hydroxyamino acids involved in thioether amino acid formation were replaced by nonhydroxyamino acids (S3N and S19A); (ii) C residues involved in thioether bridging were deleted (Δ C21, C22 and Δ C22); or (iii) a ring amino acid was replaced by an amino acid having a completely different character (G10E and Y20G). A strong decrease in production was observed when S residues involved in thioether amino acid formation were replaced by T residues (S16T and S19T). A number of conservative changes at positions 6, 12, and 14 on the Gdm backbone were tolerated and led to analogs that had altered biological properties, such as enhanced antimicrobial activity (L6V) or a remarkable resistance to proteolytic degradation (A12L and Dhb14P). The T14S substitution led to simultaneous production of two Gdm species formed by incomplete posttranslational modification (dehydration) of the S-14 residue. The fully modified Dhb14Dha analog exhibited antimicrobial activity similar to that of Gdm, whereas the Dhb14S analog was less active. Both peptides were more sensitive to tryptic cleavage than Gdm was.

The tetracyclic antimicrobial peptides epidermin (Epi) (1) and gallidermin (Gdm) (23) are structural analogs that are produced by *Staphylococcus epidermidis* Tü3298 and *Staphylococcus gallinarum* Tü3928, respectively. On the basis of their screw-like molecular structures and the presence of unusual amino acids, such as lanthionine, these compounds are classified as subgroup A lantibiotics (21). Epi and Gdm differ by only a single amino acid in the N terminus (I-6 in Epi, L-6 in Gdm). Other well-known subgroup A lantibiotics are nisin (18), subtilin (17), and Pep5 (24) (for reviews see references 9 and 21). Gdm and Epi exhibit activity against a variety of pathogenic gram-positive bacteria, such as staphylococci, streptococci, and *Propionibacterium acnes* (23), and therefore may have therapeutic value. Like most subgroup A lantibiotics, they express their antimicrobial activities mainly by forming membrane-potential-dependent pores in bacterial cytoplasmic membranes (5, 41).

As determined by cloning and sequencing of their structural genes, Epi and Gdm are ribosomally synthesized as precursor peptides consisting of a 30-amino-acid α -helical leader sequence and a 22-amino-acid C-terminal prolanthibiotic region (45, 46). Their characteristic unusual amino acids, such as 2,3-unsaturated didehydrobutyrine and the thioether amino acids (2*S*, 6*R*)-*meso*-lanthionine, (2*S*, 3*S*, 6*R*)-3-methylanthionine, and *S*-[(*Z*)-2-aminovinyl]-*D*-cysteine, are formed by a

series of posttranslational modification reactions at S, T, and C residues within the prolanthibiotic part of the precursor molecule (46). Dehydration of the S and T residues leads to the formation of didehydroalanine (Dha) and didehydrobutyrine (Dhb) residues, respectively. This reaction is most likely the first modification step, since dehydrated precursor peptides of Pep5 have been isolated recently (49). We assume that lanthionine and 3-methylanthionine residues are formed by the addition of thiol groups of C-terminal C residues to the double bonds of N-terminal Dha and Dhb residues.

Gdm and Epi contain an unsaturated C-terminal *S*-(2-aminovinyl)-*D*-cysteine residue, a unique structure among lantibiotics (21). Only mersacidin contains a similar C-terminal *S*-(2-aminovinyl)-2-methyl-*D*-cysteine residue (26). Formation of the *S*-(2-aminovinyl)-*D*-cysteine residue involves an oxidative decarboxylation reaction catalyzed by the flavoprotein EpiD (31, 33). EpiD catalyzes oxidative decarboxylation of the last C residue of unmodified pre-Epi (32). Even a synthetic heptapeptide of the C terminus of pre-Epi reacts with EpiD. A heptapeptide analog with a C-terminal ethyl-thioether structure, however, is not a substrate of EpiD (32). This suggests that the *S*-(2-aminovinyl)-*D*-cysteine residue is probably formed by the addition of the thiol group of a C-terminally generated enethiol structure to the double bond of the Dha-19 residue rather than by oxidative decarboxylation of a C-terminal lanthionine residue (46). Thus, during maturation of Epi and Gdm, oxidative decarboxylation of the last C residue may occur simultaneously with dehydration of the hydroxyamino acids or may even be the first modification step.

* Corresponding author. Mailing address: Mikrobielle Genetik, Universität Tübingen, Waldhäuser Strasse 70/8, 72076 Tübingen, Germany. Phone: 49-7071-294636. Fax: 49-7071-295937.

The maturation of Epi is terminated by extracellular cleavage of the leader peptide by EpiP (14). The Epi structural gene (*epiA*) and the genes encoding the modifying enzymes (*epiB*, *epiC*, *epiD*) are located in an operon on 54-kb plasmid pTü32 of *S. epidermidis* Tü3298 (3, 44). Transcription of this operon is positively regulated by the regulator protein EpiQ (38), whose gene (*epiQ*) is in an orientation opposite that of the *epiABCD* operon and is cotranscribed with *epiP*, the gene that encodes the processing protease. In contrast, *gdmA* is chromosomally encoded in *S. gallinarum* Tü3928 (45). The structural genes *gdmA* and *epiA* exhibit a level of nucleotide sequence identity of 85% (45).

Therefore, in this study, we developed an expression system for producing Epi and Gdm analogs in which an *epiA* mutant (EMS6) of the Epi-producing strain, *S. epidermidis* Tü3298, was used as the expression host. Epi and Gdm analogs were produced by performing site-directed mutagenesis with the structural genes to generate new analogs that had altered or new biological properties and to obtain more information about the importance of the unusual amino acids for biosynthesis and biological activity. Several new Gdm analogs that had altered biological properties were isolated and studied.

MATERIALS AND METHODS

Bacterial strains, vectors, and culture conditions. Epi-producing *S. epidermidis* Tü3298 (1) and Gdm-producing *S. gallinarum* Tü3928 (23) were used as the sources of *epiA* and *gdmA*, respectively. *Escherichia coli* WK-6 [Δ (*lac-proAB*) *galE* *strA* *NaI*^r *F'* *lacI*^r *lacZ* Δ M15 *proAB*], *E. coli* WK-6 *mutS* (WK-6 *mutS215::Tn10*) (51), helper phage M13KO7 (Pharmacia, Freiburg, Germany), and the twin phagemid system consisting of pMa5-8 and pMc5-8 were used for site-specific mutagenesis by the gapped duplex DNA (gdDNA) method (29). *Staphylococcus carnosus* TM300 (15) was used as an intermediate cloning host. *S. epidermidis* Tü3298/EMS6 (*S. epidermidis* EMS6), an *epiA* mutant of the Epi-producing strain (3), was used to express natural and mutated Gdm and Epi species. *Staphylococcal* vector pT181mcs and *E. coli-Staphylococcus* shuttle vector pCU1 (3) were used as expression vectors for natural and mutated *gdmA* and *epiA* genes. *E. coli* and *Staphylococcus* strains were cultivated in B medium (10 g of casein hydrolysate 140 [Gibco BRL] per liter, 5 g of yeast extract [Gibco BRL] per liter, 5 g of NaCl per liter, 1 g of glucose per liter, 1 g of K₂HPO₄ per liter; pH 7.2) at 37°C.

DNA preparation, transformation, and molecular cloning techniques. *Staphylococcal* DNA was prepared as described previously (50). *E. coli* supercoiled plasmid DNA was prepared by a modified alkaline lysis method (10). *E. coli* was transformed by using CaCl₂ (4), *S. carnosus* was transformed by protoplast transformation (16), and *S. epidermidis* was transformed by electroporation (2). Unless indicated otherwise, molecular cloning techniques were performed by using previously described methods (42).

Site-specific mutagenesis by the gdDNA method. Site-specific mutagenesis of *gdmA* was performed by a modified gdDNA method (29). For mutagenesis, the 1.6-kb *Sall-EcoRI* chromosomal fragment of *S. gallinarum* (encoding *gdmA* and parts of *gdmB* and *gdmT*) was cloned into the polylinker regions of phagemids pMa5-8 (conferring ampicillin resistance) and pMc5-8 (conferring chloramphenicol resistance), forming pMagdMA and pMcgdMA, respectively. The size and structure of the single-stranded target region for mutagenesis (gap) in the gdDNA intermediate and the enzymes used for in vitro DNA polymerization and ligation reactions were critical factors for efficient *gdmA* mutagenesis (data not shown). Only by reducing the original gap size, 1.6-kb (*Sall-EcoRI* fragment), to 544 bp (*ScaI* fragment) (Fig. 1) and by using T4 DNA polymerase for in vitro gap filling was mutagenesis of the *gdmA* gene performed successfully. The highest mutagenesis efficiency (96%) was achieved by using the following alterations of the protocol described previously (29).

To construct the gdDNA intermediate, single-stranded DNA from recombinant phagemid pMagdMA was isolated after infection of *E. coli* WK-6 with helper phage M13KO7 and annealed with *ScaI* vector fragments of pMcgdMA. The reaction mixture was incubated at 95°C for 5 min, allowed to cool slowly to 60°C, and kept at room temperature for 5 to 10 min. To hybridize the mutagenizing primer to the gdDNA, the hybridization mixture was incubated at 70°C for 5 min and allowed to cool slowly to room temperature.

For in vitro gap filling and the sealing reaction (in which the mutagenizing primer was incorporated into the pMcgdMA-derived strand of the gdDNA), 1 U of T4 DNA polymerase (New England BioLabs) and 10 U of *E. coli* DNA ligase (New England BioLabs) were used. The reaction mixture was incubated for 5 min at 0°C and for 20 min at 25°C. After 4 μ g of T4 gp32 (Pharmacia) was added, the mixture was incubated at 25°C for 90 min. The reaction was stopped by adding 1.2 μ l of 500 mM EDTA (pH 8.0), and the reaction mixture was kept on

ice until it was used to transform *E. coli* WK-6 *mutS*, which allowed the heteroduplex molecules to segregate into a mixed phagemid DNA population. The phagemids were transformed in *E. coli* WK-6, and transformants harboring mutated pMcgdMA derivatives were selected with 40 μ g of chloramphenicol per ml. To simplify identification of clones that had the desired mutations prior to sequencing, most of the mutagenizing primers were designed with an additional restriction site (silent mutation). The primers used for site-specific mutagenesis of the *gdmA* gene are listed in Table 1.

PCR site-specific mutagenesis. PCR site-specific mutagenesis of the *epiA* gene was performed by using pTepi14 (3) as the template and the primers listed in Table 1. For each PCR approximately 0.1 μ g of pTepi14 was used in a 100- μ l (total volume) reaction mixture; the reaction mixture contained 2.5 U of Vent DNA polymerase (New England BioLabs), 10 μ l of Vent DNA polymerase buffer (New England BioLabs), 1 μ l of bovine serum albumin (10 mg/ml; New England BioLabs), 1 mM MgCl₂, each deoxynucleoside triphosphate at a concentration of 250 μ M, and 100 pmol of each primer and was covered with 100 μ l of light mineral oil. The PCR was performed for 30 cycles, with each cycle consisting of denaturation at 93°C for 2 min, primer annealing at 42°C for 1.5 min, and extension at 72°C for 1.5 min.

DNA sequence analysis. To identify the desired mutation and verify sequence integrity, the relevant sequences of the recombinant plasmids (segments that were exposed as single-stranded DNA or were amplified by PCR) were sequenced by performing double-stranded DNA sequencing (11) by the dideoxy chain termination method (43). Sequencing reactions were performed either with a T7 Sequencing Kit (Pharmacia) and ³⁵S-dATP (Amersham) or with fluorescein-labeled primers, an AutoRead sequencing kit, and an A.L.F. DNA sequencer (Pharmacia).

Plate diffusion assay to detect Gdm and Epi species with antimicrobial activity. Plate diffusion assays were performed as described previously (3) by using *Micrococcus luteus* ATCC 9341 as the indicator strain. *S. epidermidis* EMS6 clones were spread onto the agar surfaces. Production of Gdm, Epi, or analogs of these antibiotics was detected by halo formation.

Analytical purification procedure for Gdm and Epi species. To rapidly isolate and analyze Gdm and Epi analogs and to detect inactive analogs, we developed a small-scale purification procedure in which the SMART system (Pharmacia) was used.

B medium (20 ml) was inoculated with 50 μ l of an overnight culture of an *S. epidermidis* EMS6 clone and incubated with aeration at 37°C for 24 h. Cells were removed by centrifugation, and antibiotics were isolated from the culture supernatant (15 ml) by extraction with 0.5 volume of 1-butanol. The extract was lyophilized, dissolved in 1 ml of IEX buffer A (10 mM sodium phosphate buffer, 30% [vol/vol] acetonitrile; pH 3.5), and subjected to cation-exchange chromatography with Hitrap SP columns (bed volume, 1 ml; Pharmacia) equilibrated with IEX buffer A. After the column was washed with 5 bed volumes of 5% IEX buffer B (10 mM sodium phosphate buffer, 0.5 M NaCl, 30% [vol/vol] acetonitrile; pH 3.5), the antibiotics were eluted with 5 bed volumes of 20% IEX buffer B. Fractions from each step were pooled and dried in a vacuum concentrator.

For high-performance liquid chromatography (HPLC) analysis, the fractions were resuspended in 1 ml of RPC buffer A (H₂O, 0.1% [vol/vol] trifluoroacetic acid) and applied to a μ RPC C2/C18 SC2.1/10 column (bed volume, 0.35 ml; Pharmacia) equilibrated with RPC buffer A. After the column was washed with 2.0 ml of 20% RPC buffer B (acetonitrile, 0.1% [vol/vol] trifluoroacetic acid), the peptides were eluted in the first 4.4 ml by using a flow rate of 200 μ l/min and a linear gradient consisting of 20 to 50% RPC buffer B. The *A*₂₁₄, *A*₂₆₀, and *A*₂₈₀ values were determined simultaneously. Peptides were collected by the peak fractionation method. Fractions were tested for antimicrobial activity by performing plate diffusion assays, dried with a vacuum concentrator, and stored at 4°C. Peaks that exhibited antimicrobial activity were dissolved in 30% acetonitrile and analyzed by electrospray mass spectrometry (ES-MS).

Preparative purification of natural and mutated Gdm species. Gdm species were purified from culture supernatants of producing strains by 1-butanol extraction (see above). Aliquots of lyophilized butanol extracts were dissolved in 50 ml of IEX buffer A and applied to an SP-Sepharose Fast Flow column (bed volume, 52 ml; Pharmacia). After the column was washed with 5 bed volumes of 5% IEX buffer B, natural or mutated Gdm was eluted in the first 5 bed volumes by using a flow rate of 1.5 ml/min and a linear gradient consisting of 5 to 50% IEX buffer B. Elution was monitored by determining the *A*₂₈₀. Fractions containing Gdm were pooled and diluted until the acetonitrile content was 12%. Then 0.1 volume of 1% (vol/vol) trifluoroacetic acid was added, and 100-ml aliquots were loaded onto a PepRPC 16/10 column (bed volume, 20 ml; Pharmacia) equilibrated with RPC buffer A. After the column was washed with 5 bed volumes of 20% RPC buffer B, natural or mutated Gdm was eluted in the first 11 bed volumes by using a flow rate of 2 ml/min and a linear gradient consisting of 20 to 45% RPC buffer B. Elution was monitored by determining the *A*₂₈₀. Fractions containing purified Gdm species were collected, lyophilized, and stored at 4°C.

Gdm Dhb14Dha and Gdm Dhb14S, which were coproduced, could be separated completely only by performing additional reversed-phase HPLC with a Nucleosil C2 (7 μ m) column (250 by 8 mm; Grom, Herrenberg-Kayh, Germany). The peaks were separated by isocratic elution within 20 min with 24% RPC buffer B at a flow rate of 3.5 ml/min. Under these conditions, Gdm Dhb14Dha eluted slightly later than Gdm Dhb14S.

TABLE 1. Primers used for site-specific mutagenesis

Primer ^a	Sequence ^b	Description and/or mutation
Primers used for <i>gdmA</i> mutagenesis (gdDNA method)		
1	5'-CGAATTGCTAGCAAATTT(GGA)TGTA CTCTGG-3'	L6G, <i>NheI</i>
2	5'-CGAATTGCTAGCAAATTT(GTA)TGTA CTCTGG-3'	L6V, <i>NheI</i>
3	5'-CCTGGATGT(TTA)AAAACAGGAAGCTTCAATAGC-3'	A12L, <i>HindIII</i>
4	5'-GGATGTGCCAAA(TCT)GGAAGCTTCAATAGC-3'	T14S, <i>HindIII</i>
5	5'-GGATGTGCAAAA(GCT)GGTAGC-3'	T14A
6	5'-GGATGTGCCAAA(CCA)GGAAGCTTCAATAGC-3'	T14P, <i>HindIII</i>
7	5'-GCCAAAACAGGT(ACC)TTCAATAGC-3'	S16T, <i>KpnI</i>
8	5'-GCCAAAACAGGAAGCTTCAATAGC(GGC)TGTTGTTAATTC-3'	Y20G, <i>HindIII</i>
Primers used for <i>epiA</i> mutagenesis (PCR method)		
9	5'-CTAAAATAATAATTGAAAAGG-3'	N-terminal primer
10	5'-CTGAATTAACAACAATA(AGT)GTTAAA ACTACC-3'	S19T
11	5'-CTGAATTAACAACAATA(AGC)GTTAAA ACTACC-3'	S19A
12	5'-CTGAATTAACA(TCA)ATAACTGTTAAAAC-3'	C21opal (Δ C21, C22)
13	5'-CTGAATTA(TCA)ACAATAACTGTTAAAAC-3'	C22opal (Δ C22)

^a Primers were synthesized with a model 380B DNA synthesizer (Applied Biosystems) or with a Gene Assembler Plus apparatus (Pharmacia LKB) and were purified by polyacrylamide gel electrophoresis, and concentrations were determined from A_{260} (42) measured with a model DU 7500 spectrometer (Beckman).

^b The sites of mutations are indicated by boldface letters, the changed codons are in parentheses, and the introduced restriction sites (silent mutations) used for screening are underlined.

the remaining amount of each Gdm species from the peak area measured at 280 nm (the peak area of the control was defined as 100%). The reaction products were collected by the peak fractionation method, dried with a vacuum concentrator, and stored at 4°C. For ES-MS analyses, the samples were dissolved in 30% acetonitrile.

Determination of MICs. The MICs of the different Gdm species for *M. luteus* ATCC 9341, *Arthrobacter crystallopoietes*, *Corynebacterium glutamicum*, *Staphylococcus aureus* Cowan I, and *Bacillus subtilis* ATCC 6051 were determined. A 1-mg/ml stock solution and a 1:10 dilution were prepared for each Gdm species. Serial dilutions were made in 2 ml of B medium (pH 6.8) containing different concentrations of natural or mutated Gdm. The tubes were inoculated with diluted overnight cultures of the test bacteria to a final concentration of approximately 10^6 CFU/ml. Cells were cultivated with aeration for 20 h at 37°C. A_{578} values were determined immediately after inoculation and after 20 h of cultivation. The lowest concentration of a Gdm species that inhibited growth (i.e., resulted in no increase in the A_{578}) was considered the MIC. All experiments were performed in triplicate.

RESULTS

Characterization of two Epi⁻ mutants and development of a host-vector system for expression of natural and mutated *gdmA* and *epiA* genes. Using ethyl methanesulfonate mutagenesis of *S. epidermidis* Tü3298, we isolated a series of Epi⁻ mutants; two of these isolates (EMS5 and EMS6) have mutations in the *epiA* region (3). Both EMS5 and EMS6 can be complemented to an Epi⁺ phenotype by transformation with an *epiA*-carrying plasmid, indicating that other Epi-biosynthetic genes are functional. Single point mutations in *epiA* resulted in an S3N substitution (ring A) in EMS5 and a G10E substitution (ring B) in EMS6. No Epi analog or precursor

TABLE 2. Gdm and Epi analogs produced by host strain *S. epidermidis* EMS6

<i>S. epidermidis</i> EMS6 plasmid ^a	Gdm or Epi analog produced	Acetonitrile concn (%) at which the analog eluted ^b	Molecular mass of analog (Da) ^c	
			Calculated	Determined
Mutations based on GdmA (L-6)				
pTgdmA	Gdm	34.37	2,164	2,164 [H] ± 0.3
pCUgdmA-L6G	Gdm L6G	28.82	2,108	2,108 [H] ± 0.0
pCUgdmA-L6V	Gdm L6V	32.45	2,150	2,150 [H] ± 0.3
pCUgdmA-L6V/S16T	Gdm L6V/S16T	38.71	2,164	2,164 [H] ± 0.7
pCUgdmA-A12L	Gdm A12L	35.58	2,206	2,205 [H] ± 0.4
pTgdmA-T14S	Gdm Dhb14Dha	33.34	2,150	2,150 [H] ± 0.4
	Gdm Dhb14S	32.87	2,168	2,169 [H] ± 0.3
pTgdmA-T14A	Gdm Dhb14A	32.90	2,152	2,152 [H] ± 1.1
pCUgdmA-T14P	Gdm Dhb14P	33.64	2,178	2,179 [H] ± 0.8
pCUgdmA-Y20G	None			
Mutations based on EpiA (I-6)				
pTepiA-S19T	Epi S19T	34.99	2,178	2,177 [H] ± 1.8
pTepiA-S19A	None			
pTepiA- Δ C21, C22	None			
pTepiA- Δ C22	None			

^a In addition, *S. epidermidis* EMS6 harbors plasmid pTü32-6, which contains a mutated *epiA* gene (G10E exchange). This mutant does not produce any mature or precursor Epi species which can be detected by HPLC.

^b Elution values obtained from comparable analytical HPLC analyses (μ RPC C2/C18 SC2.1/10 column; SMART system; Pharmacia).

^c Molecular masses were determined by ES-MS and were calculated by using MacProMass 1.04.

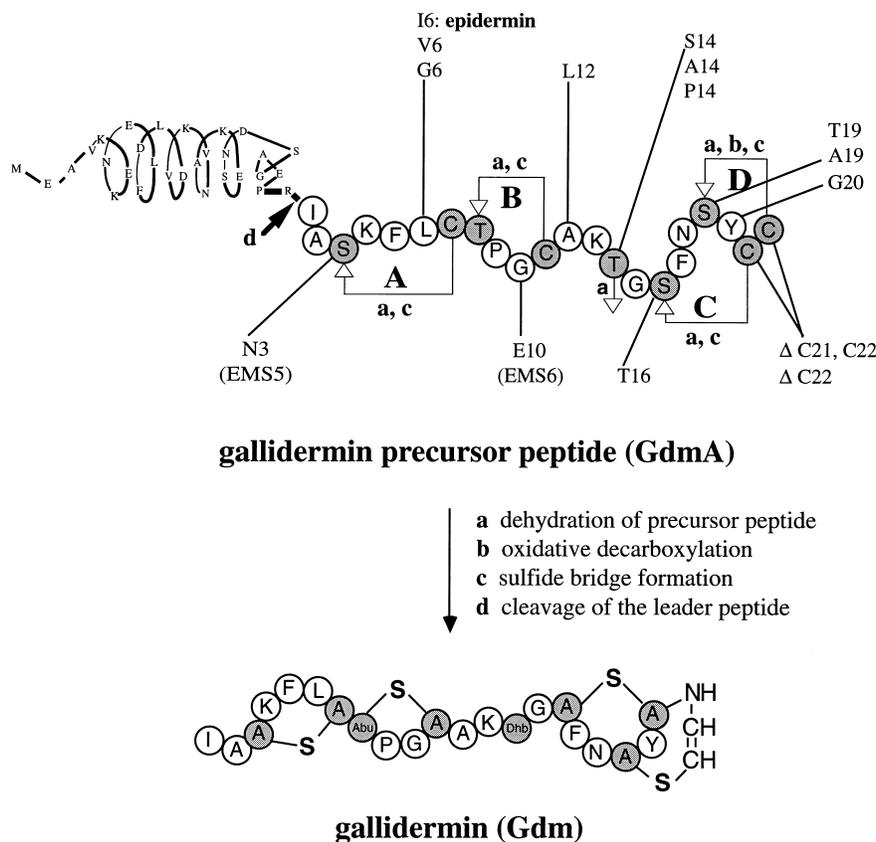


FIG. 2. Overview of maturation of pre-Gdm (GdmA) and the GdmA and EpiA analogs generated. The primary translation product of the *gdmA* gene, including the leader, is shown at the top. Mature Gdm after posttranslational modification (steps a, b, and c) and cleavage of the leader peptide (step d) is shown at the bottom. Residues that take part in posttranslational modification reactions are shaded. Rings A, B, C, and D are formed by thioether bridging. Mutations of the *epiA* gene generated by ethyl methanesulfonate mutagenesis are designated EMS5 and EMS6. (A) - S - (A) lanthionine; (Abu) - S - (A) 3-methylanthionine; Abu, 2-aminobutyric acid.

peptide was detected in the culture supernatants of EMS5 and EMS6 by analytical HPLC analyses.

We used mutant *S. epidermidis* EMS6 as an expression host for Epi and Gdm analogs. To test this mutant for its ability to synthesize the heterologous compound Gdm, a 1.6-kb *SalI-EcoRI* subfragment (encoding *gdmA* and parts of *gdmB* and *gdmT*) of the 4.8-kb *EcoRI* chromosomal fragment of *S. gallinarum* Tü3928 (45) was cloned into the polylinker region of

staphylococcal plasmid pT181mcs. The resulting plasmid, pTgdmA, was transformed into *S. carnosus* TM300, and after isolation, it was transferred into *S. epidermidis* EMS6 by electroporation. This indirect transformation method was necessary because *S. epidermidis* Tü3298 and derivative strains are poorly transformed with ligation products; only covalently closed circular plasmids were electroporated efficiently. EMS6 transformants carrying plasmid pTgdmA formed halos on *M. luteus* plates, and analyses of the culture supernatants in which analytical HPLC, ES-MS, and Edman sequencing were used revealed that only fully modified Gdm was produced (Table 2).

Plasmid pTgdmA was used to clone the mutated *gdmA* and *epiA* genes in *S. epidermidis* EMS6. pTgdmA contains two *ScaI* restriction sites that flank *gdmA* (Fig. 1); this allows replacement of *gdmA* by mutated *gdmA* genes (obtained by *ScaI* digestion of mutated pMcdgdmA derivatives derived from site-directed mutagenesis). The blunt-ended PCR fragments of mutated *epiA* were inserted into *ScaI*-cleaved pTgdmA so that the *gdmA* (-35) and *epiA* (-10) promoter regions were fused to yield a functional promoter in the resulting pTepiA derivatives. For convenient screening, the original *ScaI* sites were not reconstituted, and the nucleotide sequence downstream of *epiA* (position 361 to *ScaI* [Fig. 1]) was deleted.

E. coli-Staphylococcus shuttle vector pCU1 was also used to express mutated *gdmA* genes. The 1.6-kb *SalI-EcoRI* chromosomal fragment of *S. gallinarum* Tü3928 was cloned into the polylinker region of pCU1, and *E. coli* WK-6 was used as an

TABLE 3. Activities of Gdm and its analogs against various gram-positive indicator organisms

Gdm species	MIC ($\mu\text{g/ml}$) ^a				
	<i>M. luteus</i>	<i>A. cristallopoietes</i>	<i>C. glutamicum</i>	<i>B. subtilis</i>	<i>S. aureus</i> Cowan I
Gdm	0.004	0.005	0.065	3.0	4.0
Gdm Dhb14Dha	0.004	0.005	0.065	3.0	5.0
Gdm Dhb14S	0.008	0.01	0.13	15.0	25.0
Gdm Dhb14A	0.004	0.02	0.065	>20.0	30.0
Gdm Dhb14P	0.12	0.08	0.52	>20.0	>30.0
Gdm A12L	0.15	0.02	0.13	10.0	12.0
Gdm L6G	0.008	0.04	0.13	8.0	30.0
Gdm L6V	0.002	0.005	0.032	3.0	20.0

^a The MICs for *M. luteus*, *A. cristallopoietes*, and *C. glutamicum* range from 0.002 to 0.52 $\mu\text{g/ml}$ (~1 to 240 nM), whereas the MICs for *B. subtilis* and *S. aureus* range from 3.0 to ≥ 30 $\mu\text{g/ml}$ (1.4 to ≥ 14 μM).

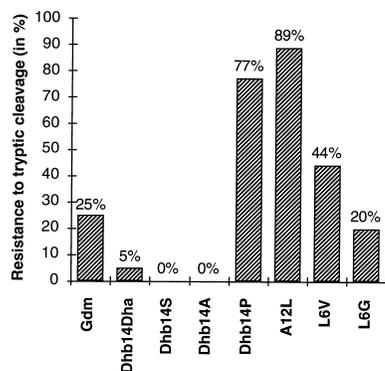


FIG. 3. Comparison of the levels of resistance to tryptic cleavage of Gdm and its analogs. Levels of resistance to tryptic cleavage were calculated from the remaining peak areas (A_{280}) obtained by a HPLC analysis after 3 h of incubation; the peak areas of uncleaved Gdm and its analogs (control) were defined as 100%.

intermediate host for cloning pCUgdmA and its derivatives. Thus, time-consuming protoplast transformation of *S. carnosus* as an intermediate host was not necessary, and transformation was greatly simplified. *S. epidermidis* EMS6 harboring pCUgdmA produced slightly less Gdm than the strain carrying pTgdmA, probably because of the reduced copy number of pCU1.

The inserted sequences (*ScaI* cassettes) of all constructions (derivatives of pTgdmA, pCUgdmA, and pTepiA) were verified by DNA sequencing.

Characterization of Gdm and Epi analogs generated by site-directed mutagenesis. An overview of the mutations generated in the Gdm and Epi precursor peptide is shown in Fig. 2. The analogs were isolated from the culture supernatants of various *S. epidermidis* EMS6 clones, purified, and analyzed by analytical HPLC, ES-MS, and continuous Edman sequencing. MICs and levels of tryptic sensitivity were determined for all Gdm analogs except Gdm L6V/S16T and Epi S19T because Gdm L6V/S16T and Epi S19T were produced in trace amounts and little material was available. The results are summarized in Tables 2 and 3.

Mutations in ring A. Since Gdm (L-6) is more active than Epi (I-6) against various gram-positive bacteria (23), we generated the following additional analogs at position 6: L6V and L6G. The production and modification of these two Gdm analogs were not impaired. The L6G analog was less active than Gdm, regardless of the indicator strain used. Compared with Gdm, the L6V analog was twice as active against *M. luteus* and *C. glutamicum*, was as active against *A. crystallopoietes* and *B. subtilis*, and was fivefold less active against *S. aureus* (Table 3). The L6G analog was almost as sensitive to trypsin as Gdm, whereas the L6V analog was more resistant (Fig. 3).

Mutations in rings C and D. To determine the importance of the C-terminal bicycle (intertwined rings C and D) for Epi and Gdm biosynthesis, we changed various amino acids in this region (Fig. 2). Using the *gdmA-L6V* gene, we created the double mutant Gdm L6V/S16T. The level of production of this analog was only 8% of the level observed for Gdm L6V. Molecular mass determinations of Gdm L6V/S16T (Table 2) revealed that the T-16 residue was dehydrated. However, we could not verify the formation of 3-methylanthionine in ring C by ES-MS. Gdm L6V/S16T exhibited antimicrobial activity; however, its MICs were not determined because of the very small amount of this analog produced.

The S19T codon was replaced in *epiA* in order to create a

posttranslationally formed *S*-(2-aminovinyl)-2-methyl-D-cysteine residue like that found in mersacidin (26). The resulting analog exhibited antimicrobial activity and was also produced in very small amounts. The amount produced was only 0.4% of the amount observed with the Gdm-producing EMS6(pTgdmA) clone (which produced approximately 12 mg of Gdm per liter). An ES-MS analysis revealed an average molecular mass of 2,177 Da for this molecule (Table 2), indicating that T-19 was dehydrated and that the C terminus was decarboxylated. We could not verify the formation of the *S*-(2-aminovinyl)-2-methyl-D-cysteine residue by ES-MS; however, thioether bridge formation probably occurred because of the observed chemical instability of the enethiol structure (31, 32).

To prevent formation of the *S*-(2-aminovinyl)-D-cysteine residue in ring D, we generated an S19A (pTepiA-S19A) substitution and a C-22 deletion (pTepiA- Δ C22). These mutations may lead to the formation of alternative thioether bridges. However, the corresponding EMS6 clones formed no halos on *M. luteus* plates, and no Epi analog or precursor form was detected by analytical HPLC. In addition, we detected no antimicrobial activity and no extracellular product when the last two C residues (pTepiA- Δ C21, C22) were deleted.

An amino acid substitution that was not directly involved in thioether bridge formation in ring D was created by replacing the bulky Y-20 residue with a G (pCUgdmA-Y20G). However, the corresponding EMS6 clone exhibited no antimicrobial activity, and no Gdm analog or precursor peptide was detected by analytical HPLC.

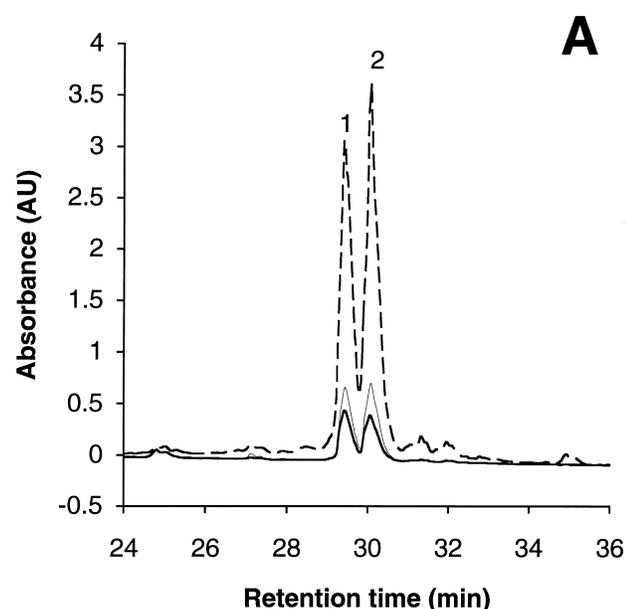
Mutations in the flexible middle region (A-12 to G-15). Using the A12L substitution, we introduced a more bulky residue into the flexible middle region (Fig. 2). The amount of this analog produced was comparable to the amount of Gdm produced; however, the activities against most of the test strains were three-fold lower (Table 3). A striking feature of this analog was its high level of resistance to tryptic cleavage (Fig. 3).

Next, we focused mainly on the 2,3-unsaturated Dhb-14 residue (Fig. 2) to investigate whether the reactive C=C double bond is important for biological activity, as suggested for the Dha-5 residue of subtilin and nisin (30, 34), or whether this residue plays a role in stabilizing a distinct structure required for pore-forming activity, as reported for the P residue in the channel-forming peptides alamethicin and melittin (48).

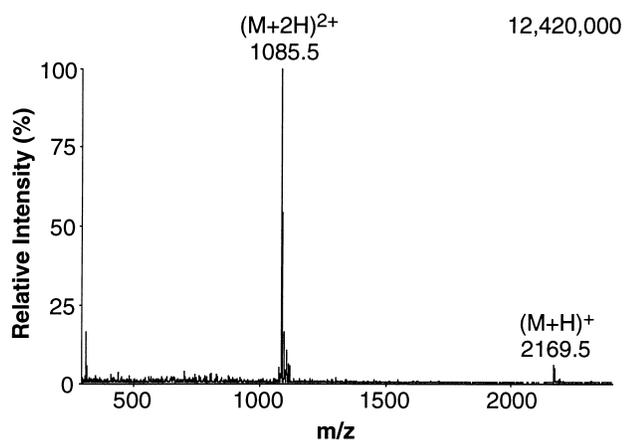
Using the T14S substitution, we produced two analogs in nearly equimolar amounts, as judged from their respective peak areas (Fig. 4A), and using ES-MS and continuous Edman degradation, we identified the two peptides as Gdm analogs that possessed either a dehydrated Dha-14 residue or an unmodified S-14 residue (Fig. 4B and C). The antimicrobial activity of Gdm Dhb14Dha was similar to that of Gdm, whereas the Dhb14S analog was less active, especially against *B. subtilis* and *S. aureus* (Table 3). Both analogs were more sensitive to tryptic cleavage than Gdm was (Fig. 3); the Dhb14S analog was one of the most sensitive analogs, and it was completely cleaved after 30 min (Fig. 5).

To remove the reactive C=C double bond, the Dhb14A analog was created. The cells produced slightly more of this analog than Gdm. The activities of Gdm Dhb14A were similar to those of Gdm against *M. luteus* and *C. glutamicum* and approximately seven-fold less against *B. subtilis* and *S. aureus* (Table 3). Like Dhb14S, the Dhb14A analog was more sensitive to tryptic cleavage than Gdm and was also completely degraded within 30 min.

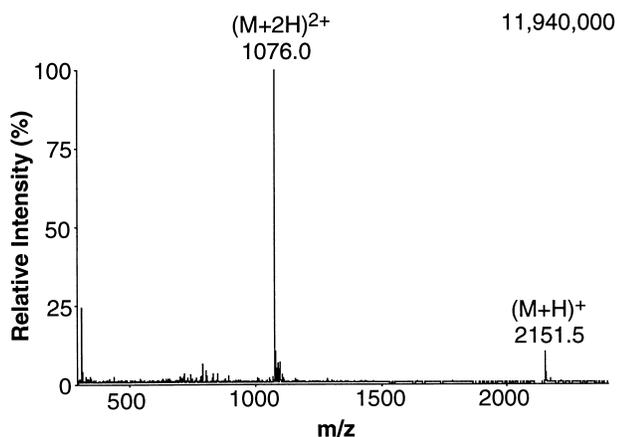
The Dhb14P substitution was expected to cause the strongest conformational change in the middle region. The amount of this analog produced was comparable to the amount of Gdm



+Profile Q1SCAN
2 peaks / HyperMass=2169[H] \pm 0.3



+Profile Q1SCAN
2 peaks / HyperMass=2150[H] \pm 0.4



produced, and the antimicrobial activities of this compound were generally 8- to 30-fold less than the antimicrobial activities of Gdm (Table 3). This analog exhibited pronounced resistance to tryptic cleavage (Fig. 3).

DISCUSSION

We demonstrated that although Epi and Gdm are produced naturally by two staphylococcal species that exhibit levels of DNA-DNA homology of only 10 to 25% (25), Gdm can be synthesized successfully by using the heterologous organism *S. epidermidis* EMS6 as the expression host and the cloned *gdmA* gene. The *gdmA* promoter was controlled by the transcriptional activator EpiQ with an efficiency similar to that of the *epiA* promoter (39), and the enzymes involved in Epi biosynthesis (including maturation and secretion) functioned efficiently with the *gdmA* gene product.

Mutagenesis at amino acid positions that are directly involved in thioether amino acid formation resulted in the loss of production or a large decrease in production. Loss of production (i.e., no HPLC-detectable extracellular product) was observed with the S19A substitution, the deletion of the C-22 residue (ring D), and the S3N substitution (ring A). Thus, we hypothesized that biosynthesis and/or secretion of Epi and Gdm is severely impaired when formation of only one of the four thioether bridges is prevented.

We also observed no production with the G10E (ring B) and Y20G (ring D) substitutions, suggesting that these mutations interfere with thioether bridge formation, although the substituted residues are not directly involved. The substitution of the G-10 residue for a bulky and negatively charged E residue may result in steric hindrance of thioether bridge formation on ring B. Two-dimensional nuclear magnetic resonance studies of Gdm (12, 13) revealed that ring B (Fig. 2), which is identical to ring B of nisin and subtilin, adopts a typical β -turn type II conformation. This specific conformation would be disturbed by a G10E exchange and, as a consequence, thioether bridge formation might be affected, regardless of whether this reaction is enzyme catalyzed or occurs spontaneously in a Michael addition-like reaction (47).

Recent investigations of the substrate specificity of EpiD (32) have revealed that a precursor molecule possessing a G residue instead of the Y-20 residue is not a substrate of EpiD. This finding suggests that at least oxidative decarboxylation of the last C residue of the mutant Y20G precursor peptide is prevented. However, production of a Gdm analog that has a C-terminal lanthionine residue should still be possible if only the EpiD reaction is affected. Thus, the absence of this analog in the culture supernatant may indicate that an additional modification reaction and/or secretion is also affected.

In summary, for all mutations leading to the loss of production, further investigations will determine whether only secretion is blocked or whether other stages of biosynthesis are also blocked. We also cannot entirely rule out the possibility that only partially modified precursor peptides are rapidly degraded by the EMS6 host.

The only thioether amino acid mutations that resulted in the production of substances that exhibited antimicrobial activity

FIG. 4. (A) Analytical reversed-phase HPLC elution profile of prepurified culture supernatant of *S. epidermidis* EMS6(pTgdmA-T14S). Peak 1, Gdm Dhb14S; peak 2, Gdm Dhb14Dha. We simultaneously determined A_{214} (dashed line), A_{260} (thin solid line), and A_{280} (thick solid line). (B and C) Electrospray mass spectra of Gdm Dhb14S (B) and Gdm Dhb14Dha (C) recorded after preparative purification. AU, absorbance units.

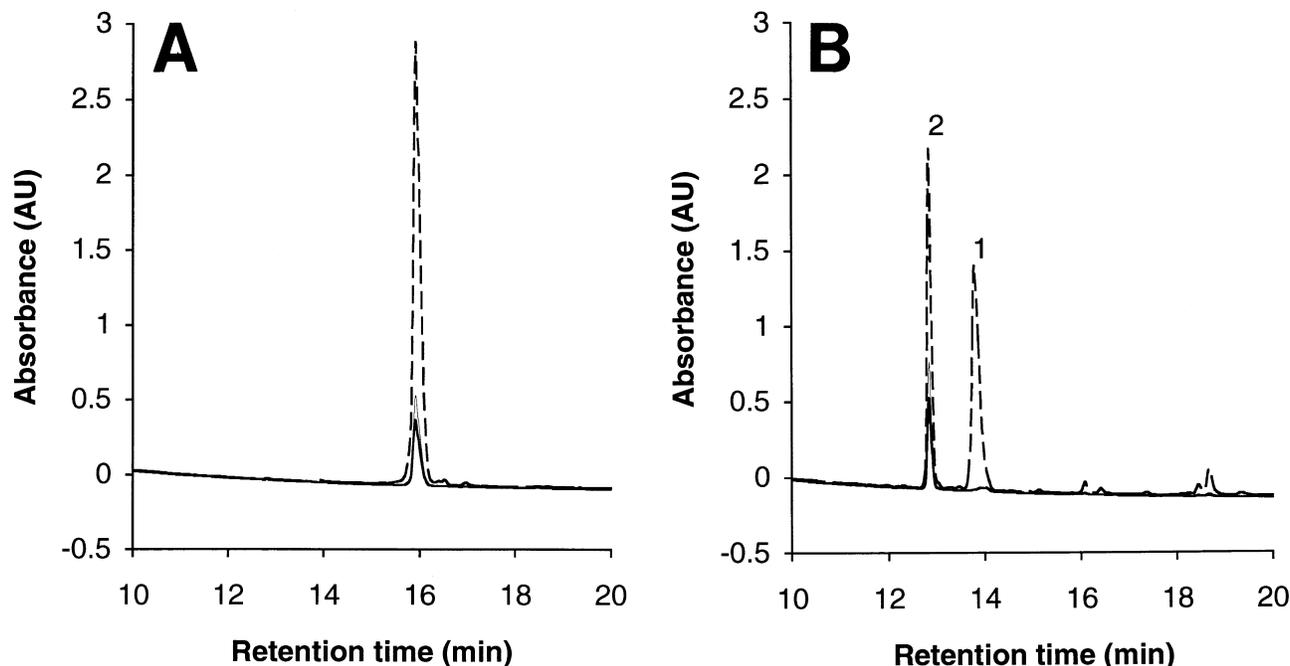


FIG. 5. Reversed-phase HPLC elution profile of the Gdm Dhb14S tryptic cleavage assay mixture. We simultaneously determined A_{214} (dashed line), A_{260} (thin solid line), and A_{280} (thick solid line). Gdm Dhb14S was incubated for 30 min in the absence (A) and in the presence (B) of trypsin. (B) Peak 1, Gdm Dhb14S¹⁻¹³ (1,302 Da); peak 2, Gdm Dhb14S¹⁴⁻²² (885 Da). The N-terminal fragment (peak 1) exhibited almost no A_{260} and A_{280} because of the lack of aromatic amino acids, which would have contributed to strong absorption at these wavelengths (19); the F-5 residue contributed only very weakly to the A_{260} . The C-terminal fragment revealed the typical absorption pattern of Gdm ($A_{260} > A_{280}$) because of the S-(2-aminovinyl)-D-cysteine residue (23, 31). AU, absorbance units.

were the S19T (ring D) and S16T (ring C) substitutions. The extremely low amounts of Epi S19T and Gdm L6V/S16T produced could be explained by assuming that dehydration of T-16 and T-19 is inefficient and that only fully modified (dehydrated and subsequently thioether-bridged) precursor peptides are efficiently secreted. The simultaneous production of Gdm Dhb14S and Gdm Dhb14Dha is an indication that the efficiencies of S dehydration and T dehydration are different. Since in natural GdmA a T-14 residue appears to be modified as efficiently as an S-16 or S-19 residue, we speculate that the dehydration efficiency for the hydroxyamino acids might depend on the location of these residues. In addition to dehydration, the efficiency of thioether bridge formation and/or secretion might also be affected by the S19T and S16T mutations. Incomplete dehydration has also been reported for nisin (G18T substitution) (30) and Pep5 (6).

A crucial characteristic for the use of peptide antibiotics is the inactivation of these molecules by proteolytic degradation. With Epi and Gdm (e.g., when they are used as therapeutic agents to treat acne), it is advantageous that the peptides naturally exhibit rather high levels of resistance to proteolytic degradation. Of the various peptidases that have been tested (pronase, trypsin, pepsin, thermolysin, collagenase, and carboxypeptidases A and B), only pronase and trypsin cleave Epi (1). Proteolytic degradation by the other peptidases is sterically hindered because of the presence of the thioether bridges. Therefore, in this study, samples of purified natural and mutated Gdm were tested for their sensitivity to tryptic cleavage in order to screen for analogs that exhibit increased resistance to proteolytic degradation and, as a consequence, a prolonged period of action.

All Gdm analogs were cleaved by trypsin only in the central part of the molecule between K-13 and the residue at position 14, which is consistent with previous reports concerning Gdm

(23) and Epi (1). Tryptic cleavage at the second putative tryptic cleavage site, the K-4-F-5 bond (ring A), is sterically hindered, at least under the conditions which we used.

Two-dimensional nuclear magnetic resonance studies of the Gdm molecule (12, 13) revealed that the rigid rings (A and B, C and D) are connected by a flexible middle region (A-12 to G-15). The conformation of this region (including the flexibility of the peptide backbone) appears to be important for antimicrobial activity and resistance to tryptic cleavage. Gdm analogs that supposedly exhibited decreased flexibility of the middle region, such as Gdm Dhb14P and Gdm A12L, were very resistant to tryptic cleavage, and the activities of these analogs against various gram-positive indicator bacteria were greatly reduced (Fig. 3 and Table 3).

According to modeling studies in which the Gdm-trypsin interaction was examined (21), the Gdm molecule must adopt a bent structure in order to fit into the catalytic cleft of the trypsin molecule. This bent structure is supported by the flexibility of the peptide backbone in the middle region. Thus, the high levels of resistance to tryptic cleavage observed with Gdm Dhb14P and Gdm A12L may reflect decreased flexibility of the middle region, which impedes formation of the bent structure.

A P residue following a K residue usually has a strong negative influence on trypsin action (22). This may help explain the high level of resistance to tryptic cleavage observed with Gdm Dhb14P (whose tryptic cleavage site is K-13-P-14). However, the negative influence of a preceding L residue is not known (22), suggesting that the large increase in the level of resistance to tryptic cleavage observed with Gdm A12L (whose tryptic cleavage site is identical to that of Gdm) is mainly caused by restricted flexibility in the middle region.

It has been reported previously that the K-13-Dhb-14 bond of Gdm is cleaved by trypsin with a strikingly lower efficiency than the bonds of K to normal protein amino acids are cleaved

(23). Thus, increased substrate affinity of the trypsin molecule and increased flexibility of the peptide backbone in the middle region, leading to greater accessibility of the tryptic cleavage site, may be responsible for the greatly reduced resistance to tryptic cleavage observed with Gdm Dhb14A and Gdm Dhb14S (whose tryptic cleavage sites are K-13-A-14 and S-14, respectively [Fig. 3 and 5]). This effect was strikingly less pronounced in Gdm Dhb14Dha (whose tryptic cleavage site is K-13-Dha-14). Interestingly, Gdm L6V and Gdm L6G differ with respect to their levels of tryptic sensitivity, although these Gdm analogs are not altered in the vicinity of the tryptic cleavage site, which may indicate that there is a slight overall change in the conformation of the L6V and L6G analogs.

When all of the Gdm derivatives altered in the middle region were compared, only the Dhb14Dha analog exhibited antimicrobial activity similar to that of Gdm (Table 3). This may indicate that the 2,3-didehydroamino acids are important for maintaining a structure required for efficient pore formation. It has also been suggested that polypeptide backbone flexibility plays a critical role in the biological activity of the channel-forming peptides alamethicin and melittin (48). However, the lower levels of antimicrobial activity observed with the Dhb14A and Dhb14S analogs might also be due to the removal of the reactive C=C double bond. With Gdm Dhb14A, decreases in antimicrobial activity were observed with only three of the five indicator strains used (Table 3). Thus, we speculate that Gdm exerts its antimicrobial activity by different mechanisms, a Dhb-Dha-dependent mechanism and a Dhb-Dha-independent mechanism. Subtilin exerts its activity against *Bacillus* spp. by two different mechanisms. The inhibitory effect on *Bacillus* spore germination is dependent on the Dha-5 residue and is most likely caused by a Michael type of reaction between the didehydro residue and nucleophilic membrane sulfhydryl groups (34). The ability to lyse vegetative *Bacillus* cells, however, is independent of the Dha-5 residue (35).

Thus, our results and the results obtained previously by mutagenesis of other type A lantibiotics (6, 30, 35) strongly indicate that the molecular mechanisms by which these peptides exert their antimicrobial activities may differ and that general function of the 2,3-didehydroamino acids for biological activity cannot be determined yet.

The test strains used varied greatly in their susceptibilities to Gdm and its analogs (Table 3), possibly because of differences in membrane phospholipid composition (20, 28) or different membrane potentials (27, 41). Both factors are crucial for the formation of voltage-dependent transmembrane pores (41). Furthermore, the composition of the bacterial cell wall might influence the kinetics of pore formation. Cationic lantibiotics interact with polyanions (e.g., teichoic acids) of the cell wall (7) and with membrane-bound cell wall precursor molecules (40). Binding of lantibiotics to these components may influence the kinetics of pore formation and may also be responsible for secondary killing mechanisms observed with Pep5 and nisin, such as activation of autolytic enzymes (7, 8) and inhibition of murein synthesis (40).

Since *B. subtilis* and *S. aureus* strains produce a variety of exoproteases (37), the strikingly lower susceptibilities of these test strains (Table 3) might be partially due to proteolytic degradation of Gdm and its analogs.

In summary, we successfully produced Gdm and Epi analogs that exhibited increased antimicrobial activities and/or increased levels of resistance to proteolytic degradation compared with Gdm. Furthermore, we obtained valuable information about the structural elements required for proper biosynthesis of Epi and Gdm. Nuclear magnetic resonance analyses of the analogs generated and black lipid membrane studies

may provide further information on the structure-activity relationship of these lantibiotics and may lead to target-oriented peptide engineering.

ACKNOWLEDGMENTS

We thank Christoph Kempter for help with the mass spectrometry analyses, Arielle Ferrandon and Christine Pannicke for expert technical assistance, and Karen A. Brune for critically reading the manuscript.

This work was supported by grant Az O1 KI 8908/5 from the Bundesministerium für Forschung und Technik.

REFERENCES

- Allgaier, H., G. Jung, R. G. Werner, U. Schneider, and H. Zähner. 1986. Epidermin: sequencing of a heterodet tetracyclic 21-peptide amide antibiotic. *Eur. J. Biochem.* **160**:9–22.
- Augustin, J., and F. Götz. 1990. Transformation of *Staphylococcus epidermidis* and other staphylococcal species with plasmid DNA by electroporation. *FEMS Microbiol. Lett.* **66**:203–208.
- Augustin, J., R. Rosenstein, B. Wieland, U. Schneider, N. Schnell, G. Engelke, K.-D. Entian, and F. Götz. 1992. Genetic analysis of epidermin biosynthetic genes and epidermin-negative mutants of *Staphylococcus epidermidis*. *Eur. J. Biochem.* **204**:1149–1154.
- Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.). 1990. *Current protocols in molecular biology*. John Wiley & Sons, Inc., New York.
- Benz, R., G. Jung, and H.-G. Sahl. 1991. Mechanism of channel-formation by lantibiotics in black lipid membranes, p. 359–372. *In* G. Jung and H.-G. Sahl (ed.), *Nisin and novel lantibiotics*. ESCOM Science Publishers, Leiden, The Netherlands.
- Bierbaum, G., M. Reis, C. Szekat, and H.-G. Sahl. 1994. Construction of an expression system for engineering of the lantibiotic Pep5. *Appl. Environ. Microbiol.* **60**:4332–4338.
- Bierbaum, G., and H.-G. Sahl. 1987. Autolytic system of *Staphylococcus simulans* 22: influence of cationic peptides on activity of *N*-acetylmuramoyl-L-alanine amidase. *J. Bacteriol.* **169**:5452–5458.
- Bierbaum, G., and H.-G. Sahl. 1988. Influence of cationic peptides on the activity of the autolytic endo- β -*N*-acetylglucosaminidase of *Staphylococcus simulans* 22. *FEMS Microbiol. Lett.* **58**:223–228.
- Bierbaum, G., and H. G. Sahl. 1993. Lantibiotics—unusually modified bacteriocin-like peptides from Gram-positive bacteria. *Zentralbl. Bakteriol.* **278**:1–22.
- Birnboim, H. C., and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* **7**:1513–1523.
- Chen, E. J., and P. H. Seeburg. 1985. Supercil sequencing: a fast and simple method for sequencing plasmid DNA. *DNA* **4**:165–170.
- Freund, S., G. Jung, O. Gutbrod, G. Folkers, and W. A. Gibbons. 1991. The three-dimensional solution structure of gallidermin determined by NMR-based molecular graphics, p. 91–102. *In* G. Jung, and H.-G. Sahl (ed.), *Nisin and novel lantibiotics*. ESCOM Science Publishers, Leiden, The Netherlands.
- Freund, S., G. Jung, O. Gutbrod, G. Folkers, W. A. Gibbons, H. Allgaier, and R. Werner. 1991. The solution structure of the lantibiotic gallidermin. *Biopolymers* **31**:803–811.
- Geißler, S., and T. Kupke (Universität Tübingen, Tübingen, Germany). 1995. Personal communication.
- Götz, F. 1990. *Staphylococcus carnosus*. A new host for gene cloning and protein production. *J. Appl. Bacteriol. Symp. Suppl.* **69**:49–53.
- Götz, F., and B. Schuhmacher. 1987. Improvements of protoplast transformation in *Staphylococcus carnosus*. *FEMS Microbiol. Lett.* **40**:285–288.
- Gross, E., H. H. Kiltz, and E. Nebelin. 1973. Subtilin. VI. Die Struktur des Subtilins. *Hoppe-Seyler's Z. Physiol. Chem.* **354**:810–812.
- Gross, E., and J. L. Morell. 1971. The structure of nisin. *J. Am. Chem. Soc.* **93**:4634–4635.
- Hancock, W. S., and D. R. K. Harding. 1984. Detection methods suitable for monitoring the separation of amino acids, peptides and proteins, p. 189–192. *In* W. S. Hancock (ed.), *CRC Handbook of HPLC for the separation of amino acids, peptides and proteins*. CRC Press, Inc., Boca Raton, Fla.
- Jack, R., R. Benz, J. Tagg, and H.-G. Sahl. 1994. The mode of action of SA-FF22, a lantibiotic isolated from *Streptococcus pyogenes* strain FF22. *Eur. J. Biochem.* **219**:699–705.
- Jung, G. 1991. Lantibiotics—ribosomally synthesized biologically active polypeptides containing sulfide bridges and α , β -didehydroamino acids. *Angew. Chem. Int. Ed. Engl.* **30**:1051–1068.
- Keil, B. 1992. *Specificity of proteolysis*. Springer-Verlag, Berlin.
- Kellner, R., G. Jung, T. Hörner, H. Zähner, N. Schnell, K.-D. Entian, and F. Götz. 1988. Gallidermin: a new lantionine-containing polypeptide antibiotic. *Eur. J. Biochem.* **177**:53–59.
- Kellner, R., G. Jung, M. Josten, C. Kaletta, K.-D. Entian, and H.-G. Sahl.

1989. Pep5, a new lantibiotic: structure elucidation and amino acid sequence of the propeptide. *Angew. Chem. Int. Ed. Engl.* **28**:616–619.
25. Kloos, W., K. H. Schleifer, and F. Götz. 1991. The genus *Staphylococcus*, p. 1369–1420. In A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), *The prokaryotes*, 2nd ed. Springer-Verlag, New York.
 26. Kogler, H., M. Bauch, H.-W. Fehlhaber, C. Griesinger, W. Schubert, and V. Teetz. 1991. NMR-spectroscopic investigations of mersacidin, p. 159–170. In G. Jung and H.-G. Sahl (ed.), *Nisin and novel lantibiotics*. ESCOM Science Publishers, Leiden, The Netherlands.
 27. Kordel, M., and H.-G. Sahl. 1986. Susceptibility of bacterial, eukaryotic and artificial membranes to the disruptive action of the cationic peptide Pep 5 and nisin. *FEMS Microbiol. Lett.* **34**:139–144.
 28. Kordel, M., F. Schüller, and H. G. Sahl. 1989. Interaction of the pore forming-peptide antibiotics Pep 5, nisin and subtilin with non-energized liposomes. *FEBS Lett.* **224**:99–102.
 29. Kramer, W., and H.-J. Fritz. 1987. Oligonucleotide-directed construction of mutations via gapped duplex DNA. *Methods Enzymol.* **154**:350–367.
 30. Kuipers, O. P., H. S. Rollema, W. M. G. J. Yap, H. J. Boot, R. J. Siezen, and W. M. de Vos. 1992. Engineering dehydrated amino acid residues in the antimicrobial peptide nisin. *J. Biol. Chem.* **267**:24340–24346.
 31. Kupke, T., C. Kempter, V. Gnau, G. Jung, and F. Götz. 1994. Mass spectroscopic analysis of a novel enzymatic reaction: oxidative decarboxylation of the lantibiotic precursor peptide EpiA catalyzed by the flavoprotein EpiD. *J. Biol. Chem.* **269**:5653–5659.
 32. Kupke, T., C. Kempter, G. Jung, and F. Götz. 1995. Oxidative decarboxylation of peptides catalyzed by flavoprotein EpiD: determination of substrate specificity using peptide libraries and neutral loss mass spectrometry. *J. Biol. Chem.* **270**:11282–11289.
 33. Kupke, T., S. Stevanovic, H.-G. Sahl, and F. Götz. 1992. Purification and characterization of EpiD, a flavoprotein involved in the biosynthesis of the lantibiotic epidermin. *J. Bacteriol.* **174**:5354–5361.
 34. Liu, W., and J. N. Hansen. 1992. Enhancement of the chemical and antimicrobial properties of subtilin by site-directed mutagenesis. *J. Biol. Chem.* **267**:25078–25085.
 35. Liu, W., and J. N. Hansen. 1993. The antimicrobial effect of a structural variant of subtilin against outgrowing *Bacillus cereus* T spores and vegetative cells occurs by different mechanisms. *Appl. Environ. Microbiol.* **59**:648–651.
 36. Meyer, H. E., M. Heber, B. Eisermann, H. Korte, J. W. Metzger, and G. Jung. 1994. Sequence analysis of lantibiotics: chemical derivatization procedures allow a fast access to complete Edman degradation. *Anal. Biochem.* **223**:185–190.
 37. Morihara, K., and K. Oda. 1992. Microbial degradation of proteins, p. 293–364. In G. Winkelmann (ed.), *Microbial degradation of natural products*. VCH, Weinheim, Germany.
 38. Peschel, A., J. Augustin, T. Kupke, S. Stevanovic, and F. Götz. 1993. Regulation of epidermin biosynthetic genes by EpiQ. *Mol. Microbiol.* **9**:31–39.
 39. Peschel, A., and F. Götz (Universität Tübingen, Tübingen, Germany). 1995. Personal communication.
 40. Reisinger, P., H. Seidel, H. Tschesche, and W. P. Hammes. 1980. The effect of nisin on murein synthesis. *Arch. Microbiol.* **127**:187–193.
 41. Sahl, H.-G. 1991. Pore formation in bacterial membranes by cationic lantibiotics, p. 347–358. In G. Jung and H.-G. Sahl (ed.), *Nisin and novel lantibiotics*. ESCOM Science Publishers, Leiden, The Netherlands.
 42. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 43. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467.
 44. Schnell, N., G. Engelke, J. Augustin, R. Rosenstein, V. Ungermann, F. Götz, and K.-D. Entian. 1992. Analysis of genes involved in the biosynthesis of lantibiotic epidermin. *Eur. J. Biochem.* **204**:57–68.
 45. Schnell, N., K.-D. Entian, F. Götz, T. Hörner, R. Kellner, and G. Jung. 1989. Structural gene isolation and prepeptide sequence of gallidermin, a new lantibiotic containing antibiotic. *FEMS Microbiol. Lett.* **58**:263–268.
 46. Schnell, N., K.-D. Entian, U. Schneider, F. Götz, H. Zähler, R. Kellner, and G. Jung. 1988. Prepeptide sequence of epidermin, a ribosomally synthesized antibiotic with four sulphide-rings. *Nature (London)* **333**:276–278.
 47. Toogood, P. L. 1993. Model studies of lantibiotic biogenesis. *Tetrahedron Lett.* **34**:7833–7836.
 48. Vogel, H., L. Nilsson, R. Rigler, S. Meder, G. Boheim, W. Beck, H.-H. Kurth, and G. Jung. 1993. Structural fluctuations between two conformational states of a transmembrane helical peptide are related to its channel-forming properties in planar lipid membranes. *Eur. J. Biochem.* **212**:305–313.
 49. Weil, H.-P., A. G. Beck-Sickinger, J. Metzger, S. Stevanovic, G. Jung, M. Josten, and H.-G. Sahl. 1990. Biosynthesis of the lantibiotic Pep5: isolation and characterization of a prepeptide containing dehydroamino acids. *Eur. J. Biochem.* **194**:217–223.
 50. Wieland, B., C. Feil, E. Gloria-Maercker, G. Thumm, M. Lechner, J.-M. Bravo, K. Poralla, and F. Götz. 1994. Genetic and biochemical analyses of the biosynthesis of the yellow carotenoid 4,4'-diaponeurosporene of *Staphylococcus aureus*. *J. Bacteriol.* **176**:7719–7726.
 51. Zell, R., and H.-J. Fritz. 1987. DNA mismatch-repair in *Escherichia coli* counteracting the hydrolytic deamination of 5-methyl-cytosine residues. *EMBO J.* **6**:1809–1815.