

## Construction of an Expression System for Site-Directed Mutagenesis of the Lantibiotic Mersacidin

Christiane Szekat,<sup>1</sup> Ralph W. Jack,<sup>2†</sup> Dirk Skutlarek,<sup>3</sup> Harald Färber,<sup>3</sup> and Gabriele Bierbaum<sup>1\*</sup>

*Institut für Medizinische Mikrobiologie und Immunologie der Universität Bonn<sup>1</sup> and Hygiene-Institut der Universität Bonn,<sup>3</sup> D-53105 Bonn, and Institut für Organische Chemie der Universität Tübingen,<sup>2</sup> Germany*

Received 26 December 2002/Accepted 31 March 2003

The lantibiotic (i.e., lanthionine-containing antibiotic) mersacidin is an antimicrobial peptide of 20 amino acids which is produced by *Bacillus* sp. strain HIL Y-85,54728. Mersacidin inhibits bacterial cell wall biosynthesis by binding to the precursor molecule lipid II. The structural gene of mersacidin (*mrsA*) and the genes for the enzymes of the biosynthesis pathway, dedicated transporters, producer self-protection proteins, and regulatory factors are organized in a biosynthetic gene cluster. For site-directed mutagenesis of lantibiotics, the engineered genes must be expressed in an expression system that contains all of the factors necessary for biosynthesis, export, and producer self-protection. In order to express engineered mersacidin peptides, a system in which the engineered gene replaces the wild-type gene on the chromosome was constructed. To test the expression system, three mutants were constructed. In S16I mersacidin, the didehydroalanine residue (Dha) at position 16 was replaced with the Ile residue found in the closely related lantibiotic actagardine. S16I mersacidin was produced only in small amounts. The purified peptide had markedly reduced antimicrobial activity, indicating an essential role for Dha16 in biosynthesis and biological activity of mersacidin. Similarly, Glu17, which is thought to be an essential structure in mersacidin, was exchanged for alanine. E17A mersacidin was obtained in good yields but also showed markedly reduced activity, thus confirming the importance of the carboxylic acid function at position 17 in the biological activity of mersacidin. Finally, the exchange of an aromatic for an aliphatic hydrophobic residue at position 3 resulted in the mutant peptide F3L mersacidin; this peptide showed only moderately reduced activity.

Mersacidin belongs to a group of antibacterial peptides which are designated lantibiotics because they contain the rare amino acids lanthionine and/or methylanthionine (8). In contrast to peptide antibiotics produced by nonribosomal biosynthesis, lantibiotic precursors are encoded by structural genes and the rare amino acids are synthesized by posttranslational modification of the prepeptides. The various enzymes of the biosynthetic pathway, dedicated product transporters, producer self-protection proteins, and regulatory factors are encoded in biosynthetic gene clusters, which are found either on the chromosome or on mobile elements such as plasmids or transposons (25). Recently it has been shown that the biosynthetic gene cluster of mersacidin is located on the chromosome of the producer strain *Bacillus* sp. strain HIL Y-85,54728 (1).

Since lantibiotics are gene-encoded peptides, the exchange of single amino acids by site-directed mutagenesis is possible. However, in the case of lantibiotics it is not sufficient to simply express a single engineered gene in an *Escherichia coli* expression system, since the modification machinery is indispensable for the biosynthesis of the lanthionines and other modified amino acids. In addition, lantibiotics are bacteriocins, and therefore the producer strain has to be protected against its own product by the self-protection (or immunity) factors. Systems that meet these requirements have been successfully con-

structed for the expression of the lantibiotics subtilin (20), nisin (11, 12, 17, 19), Pep5 (4), epidermin (22), and mutacin II (9).

The activity spectrum of mersacidin includes several gram-positive bacteria, with staphylococci among the most sensitive organisms. The peptide acts by inhibition of bacterial cell wall biosynthesis via interaction with lipid II, the membrane-bound monomeric cell wall precursor (6, 7). Both the crystal and solution structures of mersacidin have been solved (24, 28). Mersacidin is a compact globular molecule. In methanol as well as in the crystal, the N terminus folds back in order to interact with the carboxyl group of the glutamic acid residue at position 17. Other lantibiotics that resemble mersacidin with respect to their mode of action are actagardine and ala(0)-actagardine, which are produced by *Actinoplanes gabardinensis* and *Actinoplanes liguriae* (23, 30). Moreover, with the exception of an exchange of didehydroalanine (Dha) for Ile at position 10 in actagardine and position 16 in mersacidin, the amino acid sequence of the third ring of mersacidin and the second ring of actagardine is conserved and has been suggested to constitute the binding pocket for lipid II (33). However the activity spectrum of actagardine is markedly different from that of mersacidin; actagardine is most active against streptococci and displays only low activity against staphylococcal species.

Here we describe the development of an expression system that enables the production of engineered mersacidin peptides, as well as the properties of the first mersacidin variants.

### MATERIALS AND METHODS

**Bacterial strains and plasmids.** The strains and plasmids used for construction of the expression system are described in Table 1. The *E. coli* strains used for

\* Corresponding author. Mailing address: Institut für Medizinische Mikrobiologie und Immunologie, Sigmund-Freud-Str. 25, D-53105 Bonn, Germany. Phone: 49-228-2879103. Fax: 49-228-2874808. E-mail: bierbaum@mibi03.meb.uni-bonn.de.

† Present address: Department of Microbiology, University of Otago, Dunedin, New Zealand.

TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain(s) or plasmid	Characteristics	Reference or source
<i>Bacillus</i> sp. strain HIL Y-85,54728 TT	Producer strain of mersacidin	8
<i>M. luteus</i> ATCC4698	Indicator strain	ATCC strain collection
<i>S. pyogenes</i> I-12662	Indicator strain	This study
<i>S. carnosus</i> TM300	Cloning host	27
<i>E. coli</i> JM 83	Cloning host	31
<i>E. coli</i> 71/18, JM 109, HMS 174	Cloning hosts	26
<i>E. coli</i> 71/18 BHM <i>mutS</i>	Cloning host for site directed mutagenesis	32
pTV0	Temperature-sensitive gram-positive vector, Cm <sup>R</sup>	1
pTV0[MCS]	pTV0 carrying a multiple cloning site	15
pS16I14	pTV0 carrying S16I <i>mrsA</i> in its <i>PvuII</i> site	This study
pE17A14	pTV0 carrying E17A <i>mrsA</i> in its <i>PvuII</i> site	This study
pF3L14	pTV0[MCS] carrying F3L <i>mrsA</i>	This study

mutagenesis were grown on Luria-Bertani agar, *Staphylococcus carnosus* was maintained on blood agar or tryptone soy agar, and *Bacillus* strains were cultured on nutrient broth or tryptone soy agar, always in the presence of the appropriate antibiotics. All strains were stored at  $-70^{\circ}\text{C}$  as glycerol cultures.

**Site-directed mutagenesis.** Site-directed mutagenesis was performed with a commercial phagemid system (Altered Sites *in vitro* mutagenesis system; Serva, Heidelberg, Germany) according to the instructions of the manufacturer. The 1.14-kb *EcoRI*-*KpnI* fragment containing the structural gene of mersacidin (*mrsA*) (3) and the 5' part of *mrsRI* (1) was cloned into the multiple cloning site of the pALTER-1 vector provided in the mutagenesis kit. The mutagenic oligonucleotides employed for site-directed mutagenesis are shown in Table 2. All mutated genes were confirmed by sequencing on an A.L.F. express sequencer (Pharmacia Biotech, Freiburg, Germany) or by Sequiserve (Vaterstetten, Germany).

DNA was prepared by using Qiagen genomic tips, Qiaprep spin miniprep kits, or Qiagen plasmid minikits according to the recommendations of the supplier (Qiagen, Hilden, Germany). pTV0/pTV0[MCS] or pTV0/pTV0[MCS] recombinant plasmids carrying engineered *mrsA* genes were purified from 50 ml of *S. carnosus* culture by using the HiSpeed plasmid midikit (Qiagen). General protocols were used for cloning strategies and enzymatic DNA modification (26). Digested DNA fragments were eluted from agarose gels with the QIAquick gel extraction kit (Qiagen).

The *Bacillus* sp. and *Staphylococcus carnosus* TM300 were transformed by protoplast transformation (13, 14), while *E. coli* strains were transformed by electroporation. Clones were screened by plasmid preparations or, in the case of *Bacillus*, by PCR (Table 2) performed with AGS Gold DNA polymerase (Hybaid-AGS, Heidelberg, Germany) in a PCRExpress thermocycler (Hybaid) with genomic DNA as a template. For competitive nucleotide priming, a pair of Mut and Umut oligonucleotide primers was synthesized for each mutation. These primers served as backward primers, with the 3' terminus annealing to the base which had been chosen for mutagenesis. Therefore, the sequences of the Mut and Umut primers differed only by a single base located at the 3' end. In case of the Mut primer, this base was complementary to the mutation, whereas the Umut primer was complementary to the wild-type sequence. The two primers were included into two separate PCRs. The forward primer (primer 5') was added to both reaction mixtures and annealed to the sequence downstream of *mrsE* that is present only on the chromosome and not on the plasmid. Since *Taq* polymerase does not possess proofreading activity, the enzyme did not work efficiently with the primer whose 3' end was mismatched. Therefore, only the

primer whose 3' end paired correctly with that copy of the *mrsA* gene which was located directly downstream of *mrsE* yielded a PCR product.

**Purification of engineered peptides.** For purification of S16I mersacidin, 400 ml of culture supernatant were applied to a 35-ml XAD Serdolit AD2 (Serva) column, the column was washed with water-50% methanol containing 50 mM potassium phosphate buffer (pH 7), and the peptide was eluted with acetonitrile containing 0.1% trifluoroacetic acid. The eluate was concentrated by evaporation and applied in several runs to a POROS R2 10 column (Applied Biosystems, Lincoln, Calif.) at 3 ml/min and eluted with the following gradient (eluent A, water-0.1% trifluoroacetic acid; eluent B, acetonitrile-0.1% trifluoroacetic acid): 0 min, 10% B; 3 min, 10% B; 15 min, 30% B; 23 min, 40% B; and 25 min, 100% B. The adsorption of the eluate was monitored at 220 nm. Since S16I mersacidin could not be identified directly by mass spectrometry (MS) of the crude lyophilized fractions, the fractions were analyzed by high-pressure liquid chromatography (HPLC)-MS. Lyophilized fractions were dissolved in 30% acetonitrile, and a 20- $\mu\text{l}$  aliquot was separated on a 100- by 1-mm column of Gromsil ODS-SST (Grom Analytik+HPLC, Herrenberg, Germany) in a continuous gradient of 10 to 90% acetonitrile containing 0.1% trifluoroacetic acid over 30 min at a constant flow rate of 60  $\mu\text{l}/\text{min}$ . Eluant was fed directly into a VG Quattro II triple-quadrupole mass spectrometer (MicroMass) fitted with an electrospray ionization interface, using air as the carrier gas. The mass spectrometer was operated in positive mode, and data were collected and analyzed with the software provided by the manufacturer (MassLynx). The fractions containing S16I mersacidin were rechromatographed in order to obtain sufficient material for the determination of MICs. After lyophilization, the mass of the peptide was analyzed by MS as described above.

E17A mersacidin and F3L mersacidin were purified from 100 ml of culture supernatant, which was sterilized by filtration, lyophilized, dissolved in about 30 ml of 30% acetonitrile-0.1% trifluoroacetic acid, and applied in several runs to a POROS R2 20 column. The mass was analyzed with an API 2000 Triple Quad MS (Applied Biosystems).

For the agar diffusion assay, the culture supernatant was sterilized by filtration, and aliquots were pipetted into wells in a blood agar plate that had been overlaid with soft agar containing *Micrococcus luteus* as indicator strain.

Determination of the MICs of mersacidin and engineered mersacidin peptides was performed by use of a microtiter plate assay with half-concentrated Mueller-Hinton broth and an inoculum of  $10^5$  cells of *M. luteus* per well, which was read after 16 h of incubation at  $37^{\circ}\text{C}$ .

TABLE 2. Primers used in this study

Primer	Sequence <sup>a</sup>	Product or function
PTV01	CACAAAAACAGGTCACAAGAAAAAC	442-bp fragment of pTV0[MCS]
PTV02	ACCCCTCTTCCATGTATTCAC	
NPTV02	AAGGAGTTAAGTATTATGACTGGG	818-bp product in absence of the plasmid
Primer 5'	GGGTATATGCGTATAAACTTATG	
<i>mrsA</i> mutS16I	5'd(GTTTGTACTCTA <u>ACTTT</u> AGAATGTATTTGTTAATT)3'	Mutagenesis
<i>mrsA</i> mutE17A	5'd(TACTCTA <u>ACTT</u> CGCATGTATTTGTTAATTG)3'	Mutagenesis
<i>mrsA</i> mutF3L	5'd(GAAGCAGCATGTAC <u>GCTT</u> ACATTGCCTGG)3'	Mutagenesis

<sup>a</sup> Mismatches of the oligonucleotides used for mutagenesis are underlined.

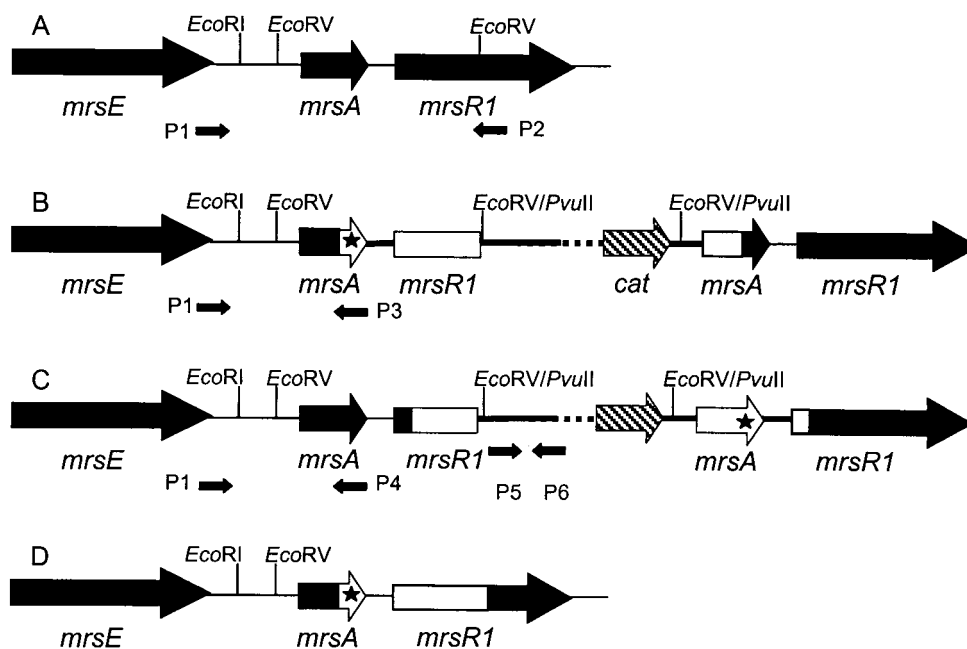


FIG. 1. Construction of clones that are produced by integration and subsequent excision of a plasmid carrying an engineered *mrsA* gene and the N terminus of *mrsR1* (white open reading frames, thick line) into the chromosome (black open reading frames, thin line). The mutation is marked by an asterisk. (A) Wild type. (B and C) The plasmid (thick line, *cat* resistance gene) has been integrated into the chromosome, and the crossover took place either upstream of the mutation (B) or downstream of the mutation (C). (D) The plasmid has been excised by the second homologous recombination step, which took place either downstream (B) or upstream (C) of the mutation in *mrsA*, resulting in an exchange of the wild-type *mrsA* gene for the engineered gene. The primers are shown by arrows: P1, primer 5'; P2, NPTV0; P3, Mut; P4, Umut; P5, PTV01; P6, PTV02.

## RESULTS

**Construction of an expression system for variant mersacidin peptides.** For expression of mutated peptides, we constructed a system that relies on replacement of the wild-type gene by the engineered gene on the chromosome. To this end, the *EcoRV* fragment carrying the engineered *mrsA* gene was ligated into the temperature-sensitive vector pTV0 or its derivative pTV0[MCS], both of which carry a chloramphenicol resistance gene, and was subcloned in *S. carnosus* at 30°C. The recombinant plasmid was then transformed into the wild-type producer strain (Fig. 1A), which was also cultured at 30°C. Since pTV0 and pTV0[MCS] replicate at a very low copy number, the colonies obtained on regeneration agar were screened by PCR with primers pTV01 and pTV02 (Table 2; Fig. 1). Subsequently, colonies which had integrated the plasmid into their chromosome were selected by a temperature shift to 45°C and growth on agar containing chloramphenicol. These colonies carried two copies of *mrsA*, the wild-type gene and the mutagenized gene, which are separated by the vector. It has to be kept in mind that the mutagenized *mrsA* gene differs from the wild-type gene by only a single or double base exchange, i.e., a point mutation. Therefore, two different kinds of mutant strains could be expected from the integration of the plasmid into the chromosome of the producer strain by a single-crossover event. When the crossover event takes place upstream of the point mutation, the mutant gene will be located downstream of *mrsE*, as shown in Fig. 1B. On the other hand, the wild-type gene will remain downstream of *mrsE* when the crossover event takes place downstream of the point

mutation, as shown in Fig. 1C. The locations of the mutant and wild-type genes were checked by competitive nucleotide priming with *Taq* polymerase. However, these clones did not show any mersacidin production, irrespective of orientation of the two *mrsA* genes. The reason for this phenomenon was not investigated, but it might be the result of a polar effect caused by the insertion of the plasmid into the biosynthetic gene cluster.

In order to obtain production of engineered peptides, we cultured the clones for about 100 generations in the absence of chloramphenicol and screened for colonies that had lost the chloramphenicol resistance by a second crossover but had kept the engineered *mrsA* gene (Fig. 1D). Colonies that had not grown on agar containing chloramphenicol after 2 days of incubation were screened by PCR with primers specific for the vector (PTV01 and PTV02), primers which anneal on the chromosome upstream and downstream of the *EcoRV* fragment containing *mrsA* (NPTV02 and primer 5'), and the specific Mut and Umut primers (Fig. 1). When the PCR indicated that the colony had integrated the engineered gene correctly into the biosynthetic gene cluster and had lost pTV0[MCS], the peptide was produced and purified as described above. The purified peptide was checked by MS for the correct modification.

**Identification of amino acid residues essential for activity of mersacidin.** The first peptide to be produced by the method described above was S16I mersacidin, which contains the Ile residue that is found in the related lantibiotic actagardine instead of the Dha residue present in mersacidin (Fig. 2). Since

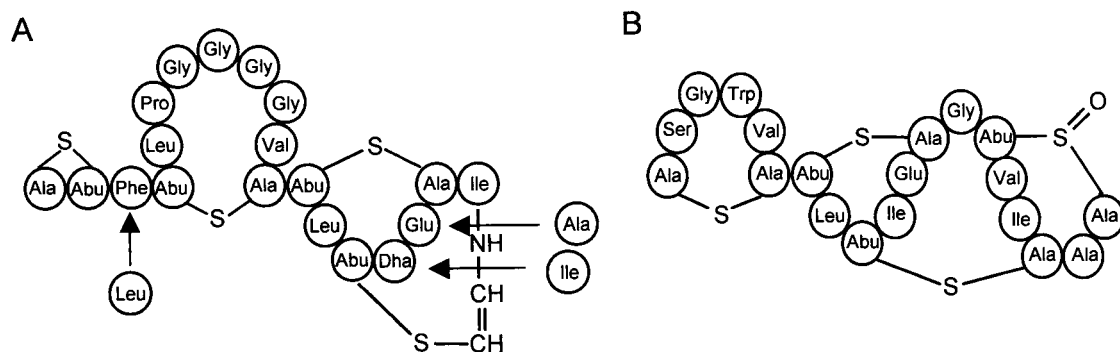


FIG. 2. (A) Structure of mersacidin (8) and exchanges that were introduced by site-directed mutagenesis. (B) Structure of the closely related lantibiotic actagardine (34).

no antibacterial activity and no mersacidin peak were detected in the supernatant of the correct clone after 72 h of culture, the peptide either was inactive, was produced only in small amounts, or was not synthesized at all. Loss of production has been described for some engineered nisin, epidermin, and Pep5 peptides (18). Therefore, the culture supernatant was fractionated on XAD and POROS R2 columns and the fractions eluting between 31 and 37% acetonitrile were screened by HPLC-MS (Fig. 3). The peptide with the correct mass of

1,869 Da was detected at 36% acetonitrile and thereafter was purified from 400 ml of culture supernatant with a yield lower than 0.5 mg/liter. The determination of the MIC showed that the activity of S16I mersacidin against *M. luteus* was nearly 1,000-fold lower than that of the wild-type peptide (mersacidin MIC, 0.1 mg/liter; S16I mersacidin MIC, 87.5 mg/liter). The antibacterial activities of HPLC fractions of culture supernatant were checked against 25 *Streptococcus* strains (belonging to Lancefield groups A, G, and B), two enterococci, and *Mi-*

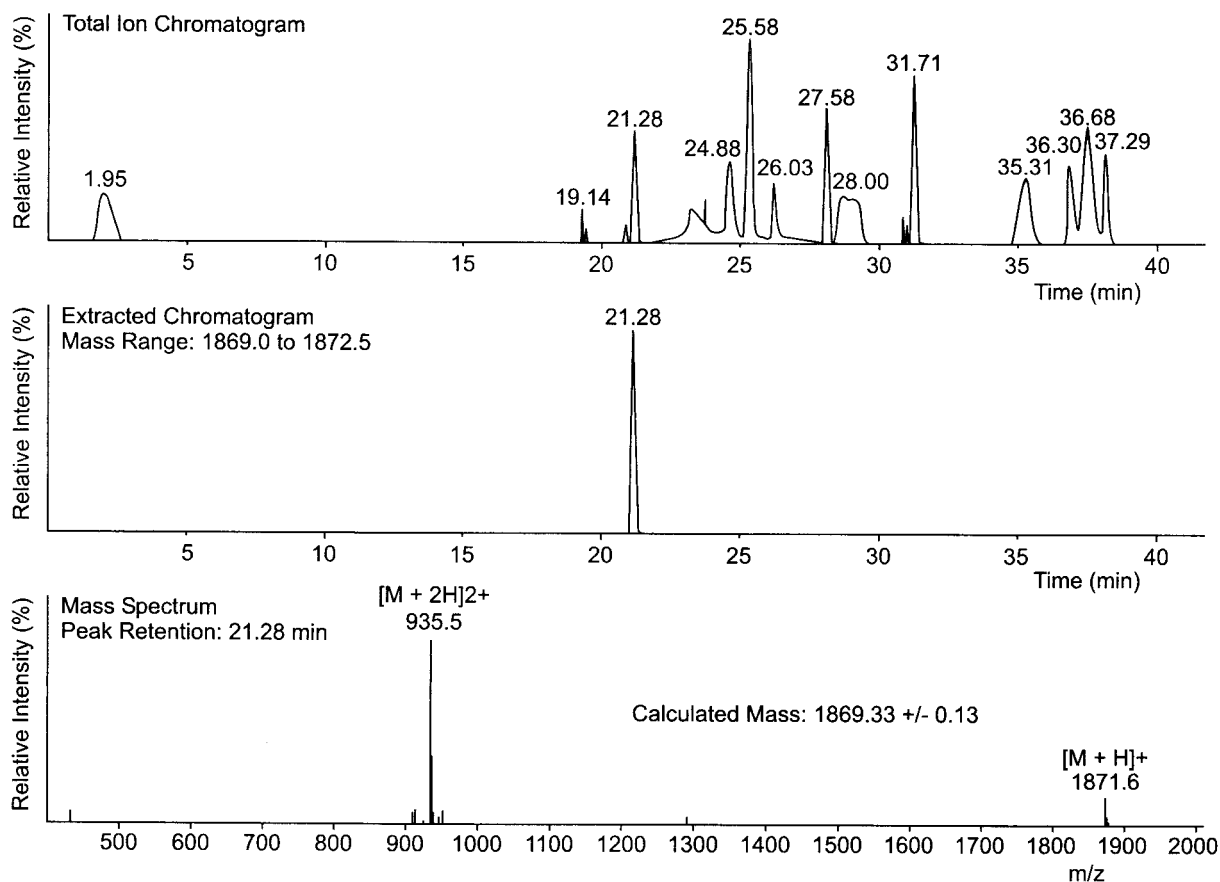


FIG. 3. HPLC-electrospray ionization-MS of S16I mersacidin. (A) Total ion chromatogram obtained after separation of the fraction containing S16I mersacidin. (B) Extracted chromatogram for peaks with masses in the range of 1869.0 to 1872.5 Da. (C) Mass determined for the peak detected at 21.28 min (expected mass for S16I mersacidin, 1,869.32 Da).

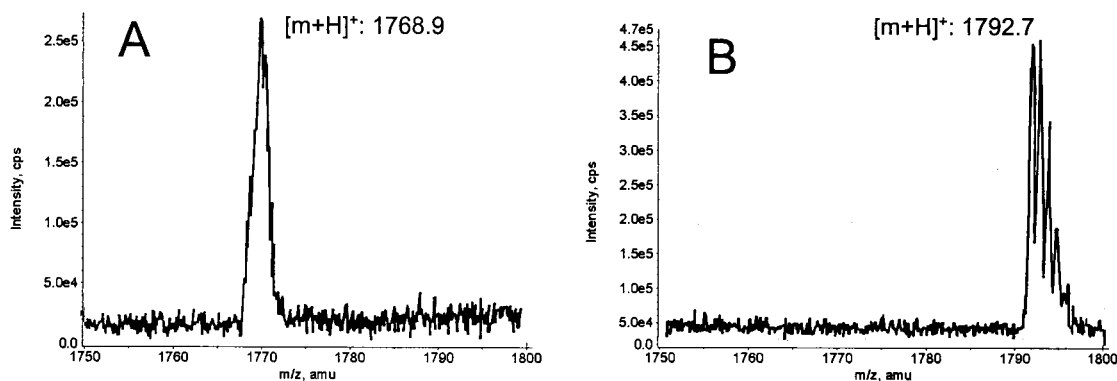


FIG. 4. MS of E17A mersacidin (expected mass, 1767.2 Da) (A) and F3L mersacidin (expected mass, 1791.2 Da) (B).

*crococcus flavus*, but no inhibition zones could be discerned on Mueller-Hinton agar plates. It was also inactive in a broth microdilution test against a clinical *Streptococcus pyogenes* isolate (I-12662) with good sensitivity to wild-type mersacidin (mersacidin MIC, 0.78 mg/liter; S16I mersacidin MIC, >25 mg/liter).

E17A mersacidin was constructed in order to assess the importance of the glutamic acid residue at position 17 for the antimicrobial activity of mersacidin. Culture supernatant of the E17A mersacidin producer did not show antimicrobial activity against *M. luteus*. However, the peptide could be identified by double detection at 220 and 266 nm during HPLC runs, since due to the NH-CH=CH-S structure in the C terminus of mersacidin, the extinction at 266 nm is about half of that at 220 nm. E17A mersacidin was produced in greater amounts than S16I mersacidin, and the purification yielded 0.41 mg of pure peptide from 100 ml of culture supernatant. The peptide with the correct mass (1,767 Da) (Fig. 4A) was inactive against *M. luteus*, with an MIC that was higher than 175 mg/liter (wild-type mersacidin MIC, 0.195 mg/liter). The yield of purified F3L mersacidin was 3.4 mg/liter of culture. The MIC of this peptide with the correct mass of 1,791 Da (Fig. 4B) against *M. luteus* was 12.5 mg/liter (wild-type mersacidin MIC, 0.195 mg/liter).

## DISCUSSION

For successful production of a mutagenized lantibiotic, the peptide must be expressed and correctly modified to contain the rare amino acids, e.g., methyllanthionine, didehydroalanine, and *S*-aminovinylmethylcysteine for mersacidin. The modification reactions are catalyzed by the modifying enzymes MrsM (dehydration and thioether formation) and MrsD (oxidative decarboxylation of Cys20) (1). In the study presented here, a gene replacement strategy was successfully employed for production of mutated mersacidin peptides. Compared to a complementation approach, it is advantageous in that the relative gene dosage of the structural gene versus the modification-export machinery (MrsD, MrsM, and MrsT) is kept at the wild-type level. Similarly, in the case of nisin yields were also higher when a gene replacement system was employed than when a complementation approach was used (11). In the present study, the rates of production of the engineered mersacidin peptides varied, but all three peptides were produced at lower rates than wild-type mersacidin, where yields of 5 to 10 mg/liter are expected. The yield was especially low for S16I mersacidin;

in this peptide, the serine residue at position 16 had been exchanged for isoleucine, thereby preventing the modification reaction of Ser16 to dehydroalanine. It is possible that this exchange and the hydrophobic side chain of the Ile residue introduced interfere with the dehydration of other residues and/or thioether formation. Therefore, the lower production rates, especially of S16I mersacidin, may be caused by inhibition of the enzymes of the modification machinery. Similar observations have also been described for other lantibiotics, especially after exchange of hydroxy amino acids; e.g., T20A Pep5 was not completely modified and was produced only at low rates (5). Furthermore, some engineered peptides that had lost hydroxy amino acids (S3N epidermin and T23A nisin A) were not produced at all (18, 22). Incomplete dehydration, indicated by the presence of peptides with molecular masses corresponding to hydrated variants (having an additional 18 Da), as found with the expression system for engineering of the lantibiotic Pep5 (4), was not seen in this system, and incompletely modified peptides were not detected. However, *Bacillus* is a potent producer of extracellular proteases, and one function of the ring structures in lantibiotics is stabilization of the peptides against the activity of proteases (5). An incompletely modified mersacidin peptide, lacking the stabilizing ring structures, would most probably be quickly degraded by one of the numerous extracellular proteases of the producer strain. This phenomenon may also contribute to the low yields of S16I mersacidin.

The exchanges S16I and E17A are situated in the third ring of mersacidin, which is conserved between mersacidin and the lantibiotic actagardine from *A. gabardinensis* (Fig. 2) (8, 34) and includes the conserved Glu residue. Actagardine also inhibits cell wall biosynthesis (29) and is able to strongly inhibit the binding of mersacidin to *M. luteus* cells, indicating that both lantibiotics compete for a similar binding site (6). However, actagardine and its variant Ala(0)-actagardine show a different activity spectrum from mersacidin (2, 30). Whereas mersacidin displays high antibacterial activity against *M. luteus* and *Staphylococcus aureus*, actagardine and its variant show good activity against streptococci. The three-dimensional structure of actagardine was elucidated by nuclear magnetic resonance (33) and seems to be rather rigid. The only hydrophilic amino acids of actagardine, Ser2 and Glu11, are part of a putative binding pocket that is formed by Ser2-Ala1-S-Ala6- $\alpha$ -aminobutyric acid 7 (Abu7)-S-Ala12-Glu11. This structure is

located in the part of actagardine that is conserved compared to mersacidin, and it was speculated that these residues might form the binding site for the antibacterial target of mersacidin and actagardine, the cell wall precursor lipid II (6, 33). In the conformation of mersacidin that is adopted in methanol, the N terminus is folded back into the center of the molecule and the amino group of Ala1 interacts with the carboxylate group of Glu17 (24), and in the crystal structure an additional interaction with Gly7 was observed (28). The polar interactions of Glu17 and Ala1 or Gly7 varied in their strength in the six slightly different structures that constitute the hexameric crystal, indicating a rather loose contact. This observation led to a speculative model in which the carboxylate group interacts with Ala1 or Gly7 in the crystal but upon encountering its target shifts to binding a ligand directly upon lipid II (28). Recent nuclear magnetic resonance experiments have indeed shown that mersacidin is a flexible molecule and that the three-dimensional structure that is adopted by mersacidin in methanol-water mixtures changes upon contact with dodecylphosphocholine micelles and then shifts again upon addition of lipid II. The first shift is especially strong in Abu13, Abu15, and Dha16; the second shift is especially pronounced for Abu13, which seems to act as a hinge region, and Gly7 and Glu17 (16). From these structural data, it is most likely that Glu17 is one of the key residues in mersacidin. The loss of antibacterial activity in E17A mersacidin demonstrated here confirms this hypothesis. In contrast, the low antibacterial activity displayed by S16I mersacidin was not expected from the comparison of the primary structures of mersacidin and actagardine. However, these results indicate that in mersacidin, either Dha16 is either essential for the interaction with lipid II or the presence of the bulky Ile residue in position 16 influences the position of the neighboring essential residue Glu17. Obviously, the primary structure of actagardine cannot simply be superimposed on mersacidin even if the amino acid sequence of both rings is otherwise conserved. In contrast, the comparatively better activity of F3L mersacidin shows that exchanges seem to be more compatible with antibacterial activity in the two N-terminal rings of mersacidin.

The mode of action of mersacidin and actagardine is not covered by any other antibiotics, since both substances interact with a novel target site that is present only on lipid II and do not bind to lipid I or intracellular peptidoglycan precursors (6). Although ramoplanin also binds to lipid II, this lipoglycopeptide recognizes lipid I and UDP-*N*-acetylmuramylpentapeptide as well (10), and vancomycin and teicoplanin target the terminal D-Ala-D-Ala residues of the peptide side chain of lipid II. The promising activities of the lantibiotics against *Staphylococcus* and *Streptococcus* make them lead structures for the design of novel antibiotics against these problematic pathogens. Chemical derivatives of actagardine have been synthesized (21, 30), but chemical modification is restricted to the C and N termini and the hydroxyl group of Ser5. In mersacidin, only the N terminus and the Glu residue represent reactive groups. The development of a system for site-directed mutagenesis of mersacidin is an important step towards the design of novel antibiotics, since it allows targeted exchange of amino acids in the structure of mersacidin.

## ACKNOWLEDGMENTS

This work was supported by the Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie (grant 01KI9705), the Deutsche Forschungsgemeinschaft (grants Bi 504/1-2 and Bi 504/1-3), and the BONFOR program of the Medizinische Einrichtungen, Universität Bonn.

We are grateful to Aventis Pharma GmbH (Frankfurt, Germany) for providing mersacidin and the mersacidin producer strain. We thank S. Schmitz for expert technical assistance.

## REFERENCES

1. Altena, K., A. Guder, C. Cramer, and G. Bierbaum. 2000. Biosynthesis of the lantibiotic mersacidin: organization of a type B lantibiotic gene cluster. *Appl. Environ. Microbiol.* **66**:2565–2571.
2. Arioli, V., M. Berti, and L. G. Silvestri. 1976. Gardimycin, a new antibiotic from *Actinoplanes*. III. Biological properties. *J. Antibiot.* **29**:511–515.
3. Bierbaum, G., H. Brötz, K. P. Koller, and H. G. Sahl. 1995. Cloning, sequencing and production of the lantibiotic mersacidin. *FEMS Microbiol. Lett.* **127**:121–126.
4. 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.
5. Bierbaum, G., C. Szekat, M. Josten, C. Heidrich, C. Kempter, G. Jung, and H. G. Sahl. 1996. Engineering of a novel thioether bridge and role of modified residues in the lantibiotic Pep5. *Appl. Environ. Microbiol.* **62**:385–392.
6. Brötz, H., G. Bierbaum, K. Leopold, P. E. Reynolds, and H. G. Sahl. 1998. The lantibiotic mersacidin inhibits peptidoglycan synthesis by targeting lipid II. *Antimicrob. Agents Chemother.* **42**:154–160.
7. Brötz, H., G. Bierbaum, P. E. Reynolds, and H. G. Sahl. 1997. The lantibiotic mersacidin inhibits peptidoglycan biosynthesis at the level of transglycosylation. *Eur. J. Biochem.* **246**:193–199.
8. Chatterjee, S., S. J. Lad, M. S. Phansalkar, R. H. Rupp, B. N. Ganguli, H. W. Fehlhaber, and H. Kogler. 1992. Mersacidin, a new antibiotic from *Bacillus*. Fermentation, isolation, purification and chemical characterization. *J. Antibiot.* **45**:832–838.
9. Chen, P., J. Novak, M. Kirk, S. Barnes, F. Qi, and P. W. Caufield. 1998. Structure-activity study of the lantibiotic mutacin II from *Streptococcus mutans* T8 by a gene replacement strategy. *Appl. Environ. Microbiol.* **64**:2335–2340.
10. Cudic, P., D. Behenna, J. Kranz, R. Kruger, A. Wand, Y. Veklich, J. Weisel, and D. McCafferty. 2002. Functional analysis of the lipoglycopeptide antibiotic ramoplanin. *Chem. Biol.* **9**:897.
11. Dodd, H. M., N. Horn, C. J. Giffard, and M. J. Gasson. 1996. A gene replacement strategy for engineering nisin. *Microbiology* **142**:47–55.
12. Dodd, H. M., N. Horn, Z. Hao, and M. J. Gasson. 1992. A lactococcal expression system for engineered nisins. *Appl. Environ. Microbiol.* **58**:3683–3693.
13. Götz, F., and B. Schumacher. 1987. Improvement of protoplast transformation in *Staphylococcus carnosus*. *FEMS Microbiol. Lett.* **40**:285–288.
14. Grosch, J. C., and K. L. Wollweber. 1982. Transformation of *Bacillus licheniformis* and *Bacillus amyloliquefaciens* protoplasts by plasmid DNA, p. 97–105. *In* U. N. Streips, H. S. Goodgal, W. R. Guild, and G. A. Wilson. (ed.), Genetic exchange. Marcel Dekker, New York, N.Y.
15. Guder, A., T. Schmitter, I. Wiedemann, H. G. Sahl, and G. Bierbaum. 2002. Role of the single regulator MrsR1 and the two-component system MrsR2/K2 in the regulation of mersacidin production and immunity. *Appl. Environ. Microbiol.* **68**:106–113.
16. Hsu, S. T., E. Breukink, G. Bierbaum, H. G. Sahl, B. De Kruijff, R. Kaptein, N. A. Van Nuland, and A. M. Bonvin. 2003. NMR study of mersacidin and lipid II interaction in DPC micelles: conformational changes are a key to antimicrobial activity. *J. Biol. Chem.* **278**:13110–13117.
17. Kuipers, O. P., M. M. Beerthuyzen, R. J. Siezen, and W. M. de Vos. 1993. Characterization of the nisin gene cluster *nisABTCIPR* of *Lactococcus lactis*. Requirement of expression of the *nisA* and *nisI* genes for development of immunity. *Eur. J. Biochem.* **216**:281–291.
18. Kuipers, O. P., G. Bierbaum, B. Ottenwälder, H. M. Dodd, N. Horn, J. Metzger, T. Kupke, V. Gnau, R. Bongers, P. van den Bogaard, H. Kusters, H. S. Rollema, W. M. de Vos, R. J. Siezen, G. Jung, F. Götz, H. G. Sahl, and M. J. Gasson. 1996. Protein engineering of lantibiotics. *Antonie Leeuwenhoek* **69**:161–169.
19. Kuipers, O. P., H. S. Rollema, R. Zell, 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.
20. 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.
21. Malabarba, A., R. Pallanza, M. Berti, and B. Cavalleri. 1990. Synthesis and biological activity of some amide derivatives of the lantibiotic actagardine. *J. Antibiot.* **43**:1089–1097.
22. Ottenwälder, B., T. Kupke, S. Brecht, V. Gnau, J. Metzger, G. Jung, and F.

- Götz. 1995. Isolation and characterization of genetically engineered gallidermin and epidermin analogs. *Appl. Environ. Microbiol.* **61**:3894–3903.
23. Parenti, F., H. Pagani, and G. Beretta. 1976. Gardimycin, a new antibiotic from *Actinoplanes*. I. Description of the producer strain and fermentation studies. *J. Antibiot.* **29**:501–506.
24. Prasch, T., T. Naumann, R. L. Markert, M. Sattler, W. Schubert, S. Schaal, M. Bauch, H. Kogler, and C. Griesinger. 1997. Constitution and solution conformation of the antibiotic mersacidin determined by NMR and molecular dynamics. *Eur. J. Biochem.* **244**:501–512.
25. Sahl, H. G., and G. Bierbaum. 1998. Lantibiotics: biosynthesis and biological activities of uniquely modified peptides from gram-positive bacteria. *Annu. Rev. Microbiol.* **52**:41–79.
26. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
27. Schleifer, K. H., and U. Fischer. 2002. Description of a new species of the genus *Staphylococcus*: *Staphylococcus carnosus*. *Int. J. Sys. Bacteriol.* **32**:153–156.
28. Schneider, T. R., J. Karcher, E. Pohl, P. Lubini, and G. M. Sheldrick. 2000. Ab initio structure determination of the lantibiotic mersacidin. *Acta Crystallogr. D* **56**:705–713.
29. Somma, S., W. Merati, and F. Parenti. 1977. Gardimycin, a new antibiotic inhibiting peptidoglycan synthesis. *Antimicrob. Agents Chemother.* **11**:396–401.
30. Vertesy, L., W. Aretz, A. Bonnefoy, E. Ehlers, M. Kurz, A. Markus, M. Schiell, M. Vogel, J. Wink, and H. Kogler. 1999. Ala(0)-actagardine, a new lantibiotic from cultures of *Actinoplanes liguriae* ATCC 31048. *J. Antibiot.* **52**:730–741.
31. Vieira, J., and J. Messing. 1982. The pUC plasmids, an M13mp7-derived system for insertional mutagenesis and sequencing with synthetic universal primers. *Gene* **19**:259–268.
32. 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.
33. Zimmermann, N., and G. Jung. 1997. The three-dimensional solution structure of the lantibiotic murein-biosynthesis-inhibitor actagardine determined by NMR. *Eur. J. Biochem.* **246**:809–819.
34. Zimmermann, N., J. W. Metzger, and G. Jung. 1995. The tetracyclic lantibiotic actagardine. <sup>1</sup>H-NMR and <sup>13</sup>C-NMR assignments and revised primary structure. *Eur. J. Biochem.* **228**:786–797.