Isolation, Structural Characterization, and Properties of Mattacin (Polymyxin M), a Cyclic Peptide Antibiotic Produced by *Paenibacillus kobensis* M

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**Running Title:** Characterization of Mattacin

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Summary

Mattacin is a non-ribosomally synthesized, decapeptide antibiotic produced by *Paenibacillus kobensis* M. The producing strain was isolated from a soil/manure sample and identified using 16s rRNA sequence homology along with chemical and morphological characterization. An efficient production and isolation procedure was developed to afford pure mattacin. Structure elucidation using a combination of chemical degradation, multidimensional NMR studies (COSY, HMBC, HMQC, ROESY), and mass spectrometric (MALDI MS/MS) analyses showed that mattacin is identical to polymyxin M, an uncommon antibiotic previously reported in certain *Bacillus* species by Russian investigators. Mattacin (polymyxin M) is cyclic and possesses an amide linkage between the C-terminal threonine and the side chain amino group of the diaminobutyric acid (DAB) residue at position 4. It contains a (S)-6-methyloctanoic acid moiety attached as an amide at the N-terminal amino group, one D-leucine, six L-α,γ-diaminobutyric acid (DAB), and three L-threonine residues. Transfer NOE experiments on the conformational preferences of mattacin when bound to lipid A and microcalorimetry studies on binding to lipopolysaccharide (LPS) showed that its behavior was very similar to that observed in previous studies of polymyxin B (a commercial antibiotic), suggesting an identical mechanism of action. It was capable of inhibiting the growth of a wide variety of Gram-positive and Gram-negative bacteria, including several human and plant pathogens with activity comparable to purified polymyxin B. The biosynthesis of mattacin was also briefly examined using transpositional mutagenesis by which ten production mutants were obtained, revealing a set of genes involved in production.
Introduction

To survive in the natural environment and compete with other microorganisms for resources, many bacteria produce antimicrobial compounds to inhibit or kill other competing strains, including human and animal pathogens. One subclass of these antimicrobial compounds is the antibacterial peptides, which can be divided into 2 categories based on the biosynthetic pathways by which they are generated. One group consists of gene-encoded, ribosomally synthesized peptides (bacteriocins) that typically have 30 to 60 residues, may be either unmodified or extensively post-translationally altered (i.e. lantibiotics), and are active against closely related bacteria (1,2,3). Peptides in the second class are non-ribosomal in origin and are produced by a series of condensations catalyzed by specific non-ribosomal peptide synthetases (NRPSs)\footnote{The abbreviations used are: NRPS, nonribosomal peptide synthetases; TSA, tryptic soy agar; TSB, tryptic soy broth; LB, Luria-Bertani; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; HPLC, high performance liquid chromatography; TFA, trifluoroacetic acid; R\textsubscript{t}, retention time; HH-COSY, proton-proton correlation spectroscopy; DQF-COSY, double quantum filtered correlation spectroscopy; TOCSY, total correlation spectroscopy; NOE, nuclear Overhauser effect; NOESY, NOE spectroscopy; ROESY, rotational nuclear Overhauser effect spectroscopy; TRNOE, two-dimensional transferred NOE; GC-MS, gas chromatography mass spectroscopy; MALDI-TOF, matrix assisted laser desorption ionization time-of-flight; LPS, lipopolysaccharide.} using a templated multienzyme mechanism (4,5). These NRPSs are large, multi-functional proteins composed of different modules, each of which has different domains capable of performing one step in the condensation of an amino acid onto a growing peptide chain (6). The resulting peptidic compounds often contain non-proteinaceous amino acids, including D-amino acids, hydroxy acids, or other unusual constituents (7). The peptide portion of antibiotics produced in this fashion is generally smaller than in ribosomal bacteriocins and usually has less than 20 amino acids (8).
Our interest in bacteriocins from lactic acid bacteria, both unmodified (9,10) and multi-component, post-translationally modified lantibiotics (11), has led us to examine other species of Gram-positive bacteria, such as Bacillus (12), for novel peptidic antimicrobial agents. During a screening program we found that a Paenibacillus kobensis strain isolated from a soil/manure sample produced an active modified peptide with broad activity against both Gram-positive and Gram-negative organisms, including a number of human and animal pathogens. We now report that the structure of the principal antimicrobial compound formed by this strain, mattacin, is identical to polymyxin M, an uncommon antibiotic previously reported in the Russian literature (13,14,15,16 all reports in Russian) (figure 2). Although polymyxins represent one of the earliest classes of commercially important antibiotics to be identified (17), and at least 15 unique polymyxins have been described (18), only polymyxin B is currently widely used and studied. In addition to efficient production and purification of mattacin, the present study describes its NMR solution structure and transfer NOE determination of conformational changes which occur upon binding to lipid A, and compares these to previous results reported by others (19) with polymyxin B. Isothermal titration calorimetry (ITC) was also employed to compare the binding of mattacin and polymyxin B to lipopolysaccharide (LPS), the major antigen of the outer membrane of Gram-negative bacteria. Finally, the biological potency of mattacin was assessed in comparison to polymyxin B, and the biosynthesis of mattacin was briefly examined.
Experimental Procedures

Bacterial Strains and Culture Conditions. The producer strain, *Paenibacillus kobensis* M, was isolated from a soil/manure sample mix and grown aerobically at 30 °C on tryptic soy agar (TSA) or in broth (TSB) with shaking (250 rpm). *Escherichia coli* BF2, a laboratory strain, was used as the standard sensitive strain. *E. coli* Jm2r’ was used as a gene cloning host for the recovery of the transposon-interrupted mutants. *E. coli* strains were grown aerobically at 37 °C on Luria-Bertani (LB) agar or in broth with shaking (250 rpm). All other strains used for the inhibition spectrum assay were grown in both broth and agar culture under their established optimum conditions and media. Erythromycin (1.5 µg/ml), lincomycin (20 µg/ml), and tetracycline (5 µg/ml) were used for selection of producer strain transformants. Kanamycin (20 µg/ml) was used for selection of *E. coli* Jm2r’ transformants.

Producer Strain Identification. A pair of degenerate primers were designed to amplify the 16s rRNA gene (20). The PCR product was purified from agarose using a Qiagen Gel Extraction kit (Qiagen Corp., Valencia, CA) and sequenced using an ABI Prism 373 DNA sequencer (Applied Biosystems, Foster City, CA). The resulting sequence was analyzed by homology comparison using the NCBI nucleotide Blast search database. A series of biochemical and morphological assays were then performed as described by Reva et al. (21) and Shida et al. (22) in order to confirm the identity of the bacterium determined by 16s rRNA gene sequence comparison down to the species level.

Antimicrobial Activity Monitoring During Purification. Antimicrobial activity was monitored by inhibition of indicator strain growth on agar plates. Plates were prepared by inoculating 200 ml of molten (48 °C) TSA (40 g/L) with 1.0 ml of a culture of the
indicator organism *E. coli* BF2 (.5 % inoculum). The molten agar was swirled gently and then dispensed in 20 ml aliquots onto sterile Petri plates, allowed to cool, and then stored at 4 °C. When performing activity assays, small wells (4.6 mm diameter) were made in the seeded agar plates, and 50 μL aliquots of the solutions to be tested were dispensed into each well. The plates were then incubated at 30 °C with growth of the indicator being visible within as little as three hours.

**Spectrum of Activity.** The antimicrobial spectrum was determined using a deferred inhibition assay previously described by Ahn and Stiles (23). Briefly, the producer strain was spotted onto TSA plates and incubated 24 hours at 30 °C. Molten 0.75% TSA, Luria Burtani (LB), or MRS soft agar (media used was optimum for each indicator strain) was then inoculated with 80 μl of a late log-phase indicator broth culture (approximately $10^6$ viable cells/ml) and poured onto the surface of the plate containing the producer strain colonies. These agar overlays were then incubated overnight at either 30 °C or 37 °C (according to the indicator’s optimum temperature), and the zones of inhibition were then measured. Alternatively, 10 μl of a purified, known-concentration antimicrobial peptide solution was spotted onto the surface of a TSA plate, allowed to dry, and overlaid as described above.

**Antimicrobial Peptide Production During Growth and Arbitrary Unit Definition.**

A fresh, overnight, producer strain culture (10 ml) was inoculated into 1 liter of TSB and incubated at 30 °C with stirring. At various time intervals during this incubation, 1 ml of culture was collected to determine growth phase and antimicrobial peptide production. These samples were immediately centrifuged (14000 rpm, 20 min, 4 °C) to remove the cells. The supernatant was heat treated at 65 °C for 20 min to inactivate any protease
activity. The activity of each supernatant was then determined using a two-fold dilution, agar diffusion test. The samples were serially diluted in 2-fold increments, and 20 µl of each dilution spotted onto a TSA plate. These spots were dried, and the plate overlaid and read as described above with *E. coli* BF2 as the indicator strain. An arbitrary activity unit (AU), defined as the 20 µl sample from the highest dilution that had a clear inhibition zone, was then determined for each fraction.

**DNA Preparation and Transformation.** Plasmids from *E. coli* were isolated using the Qiagen Plasmid mini kit (Qiagen Corp., Valencia, CA) as described by the manufacturer’s instructions. Plasmids from the producer strain were isolated using the method of Sambrook et al. (24), except that cells were treated with 10 mg/ml of lysozyme for 30 min at 37 °C prior to the SDS lysis step. Transformation of *E. coli* Jm2r’ was performed using CaCl₂ *E. coli* competent cell preparation according to the method of Sambrook et al. (24), and transformation of the producer strain was performed with the transposon delivery plasmid pLTRV3 (25) following the protocol of Dramsi et al (26).

**Transpositional Mutagenesis and DNA Cloning.** Producer strain transformants were inoculated into TSB and grown to an OD₆₀₀ of approximately 0.2. The cultures were then shifted to 41 °C for 4 h to force random chromosomal insertion of the transposon, creating a transpositional mutagenesis library. This library was then screened for loss of antibiotic production by colony deferred inhibition assay as described above. Confirmation of each mutant’s transposon insertion was performed by a PCR reaction using primers derived from the Tn917 sequence. Mutant chromosomal extractions showing the Tn917 PCR fragment were then digested with *Xba*I (Promega Corp., Madison, WI) followed by ligation with high concentration T4 DNA ligase (Gibson BRL
Life, where). The ligation mixtures were then used to transform *E. coli* Jm2r’. Plasmid preparations were performed on all Jm2r’ transformants and were sequenced in both the forward and reverse directions. Two primers were designed for plasmid sequencing. The first was derived from the sequence of the monoclonal site of the pLTV3 plasmid (5’CCG GGG ATC CTC TAG A3’), and the second was derived from 70 bp upstream of the *lacZ* gene on the pLTV3 plasmid (5’GTT AAA TGT ACA AAA TAA CAG CGA3’). The self-ligated plasmids were sequenced using an ABI Prism 373 DNA sequencer (Applied Biosystems, Foster City, CA) and the results analyzed by NCBI BLAST homology searches.

**Isolation of Mattacin.** In a 10 ml culture tube of TSB, a pre-culture of *Paenibacillus kobensis* M was grown for 24 hours with shaking (200 rpm) at 30 ºC. A 1 liter batch of TSB was then prepared by first passing through a column (2.5 x 30 cm) packed with 40 g of Amberlite XAD-16 resin (Sigma) to remove hydrophobic components from the media that would otherwise interfere with the isolation of the hydrophobic peptide. After sterilization (15 min at 121 ºC) and cooling, this modified TSB was inoculated with the entire 10 ml *P. kobensis* M preculture (1% inoculum). After a total growth time of 16 to 24 h at 30 ºC with shaking (200 rpm), the cells were removed by centrifugation (20 min, 8000 rpm). The supernatant was then passed through a column (2.5 x 50 cm) containing 60 g of Amberlite XAD-16 resin at a flow rate of 15 ml/min with the aid of a peristaltic pump. The column was then washed with 30 % ethanol (500 ml). The active peptide was then removed from the Amberlite column by washing with 500 ml of 70 % acid-isopropanol (pH 2 by addition of 1 M HCl). All fractions were assessed for activity using the well-plate assay described above. The active 70 % acid-isopropanol (pH 2) fraction
was evaporated to dryness by rotary evaporation and the yellow residue was redissolved/suspended in 5.0 mL of purified (milli-Q system, Millipore, Bedford, Mass.) water. This concentrated solution was next applied to a column (2.5 x 50 cm) containing G25 superfine Sephadex (Amersham Pharmacia, Uppsala, Sweden) at a flow rate of 1.0 ml/min. The column was eluted with purified (milli-Q) water overnight and 10 ml fractions were collected. Each fraction was again assayed for activity as described above. The active fractions 17-20 were then pooled, evaporated, and redissolved in 10 ml of 20% isopropanol in preparation for reverse phase HPLC. Complete isolation of mattacin required 2 separate HPLC methods both employing a C18 steel-walled column (Vydac, 10 x 250 mm, 5 μm). During the initial HPLC work, all isolatable peaks were assessed for antimicrobial activity using the aforementioned well-plate assay until the retention time of mattacin was well established. In the first method, a 1.0 ml injection was applied and a gradient of water and isopropanol (0.1% TFA), starting at 20 % and climbing to 30 % isopropanol over 25 min, was used (flow rate = 2.5 ml/min, detection at 225 nm). Using this method, most of the polar impurities were removed with mattacin eluting in a broad peak (Rt=18-20 min) somewhat later. The active fractions were pooled, evaporated to dryness, and redissolved in 6.0 ml of 45 % methanol. The second method employed the same C18 column using a gradient of water and methanol (0.1% TFA), starting at 45 % and climbing to 60 % methanol over 15 min (flow rate = 4.0 ml/min, detection at 225 nm). Under these conditions, mattacin was isolated as a single peak (Rt = 10-11 min). Using 1.0 ml sample injections, the entire sample was purified, and after pooling, evaporation of the methanol, and lyophilization, as much as 5 mg of pure mattacin was obtained as white powder from a 1 liter culture.
**Amino Acid Analysis.** Mattacin (100 µg) was hydrolyzed at 160 °C for 1 h with 100 µL 5.7 M HCl/0.1 % phenol in a sealed, evacuated tube. The solvent was removed by vacuum centrifugation (Speed-Vac), and the dried hydrolysate was redissolved in 0.2 M sodium citrate buffer (pH 2.0). Analysis was achieved through cation-exchange chromatography with a Beckman 6300 amino acid analysis instrument using a 120 x 2.5 mm ID column with post-column detection/quantitation by reaction with ninhydrin at 135 °C.

**Acetylation of Mattacin.** With the knowledge that mattacin contained threonine, it was hoped that a crystalline product, suitable for x-ray analysis, might be obtained by chemically modifying nucleophilic residues. To this end, 500 µg of mattacin was treated with 1.0 ml of pyridine/acetic anhydride (1:1) on ice. The mixture was allowed to warm to room temperature, and after 4 h a small aliquot (10 µl) was removed for mass spectrometric analysis.

**Mass Spectroscopy.** Samples for MALDI mass analysis were prepared using sinnapinic acid as matrix. Solutions containing the sample peptide were mixed in even part with a stock solution of sinnapinic acid (10 mg/ml) in 60 % acetonitrile (0.1 % TFA). A thin layer of sinnapinic acid was deposited on the surface of the gold target plate by delivery of a small droplet (0.7 µl) of a solution containing sinnapinic acid (4 mg/ml) in 50 % acetone/50 % methanol. After evaporation of the acetone/methanol, a 0.3 µl droplet of the solution containing the sample peptide-matrix mixture was deposited on top of the fresh matrix layer on the plate. The solvent was evaporated at 1 atm prior to analysis. Mass spectra were recorded with a single-stage reflectron, MALDI-TOF mass spectrometer (Applied BioSystems (Foster City, CA) API QSTAR Pulsar with an
oMALDI source) (27). Tandem MS/MS was performed using two different instruments. The QSTAR instrument, described above, was of the geometry QqTOF, where MS/MS analysis was achieved through collision-induced dissociation (CID) in the rf-only section of the mass spectrometer (Q₂) after mass selection with the Q₁ resolving quadrupole. Fragment ions were detected in the orthogonal time-of-flight section of the mass spectrometer. Ion generation was achieved through MALDI ionization using sinnapinic acid as the matrix. The QSTAR was equipped with a 20 Hz pulsed nitrogen laser operating at 337 nm. CID MS/MS analysis was completed using argon as the collision gas in Q₂. Also used in a second MS/MS analysis was a ThermoFinnegan (San Jose, CA) LCQ XP ion trap instrument equipped with a nanospray source. The peptide sample was dissolved in 1:1 methanol:water acidified with 0.2 % formic acid and loaded into a PicoTip (New Objective, Woburn, MA) nanospray tip. Static nanospray was achieved by applying ~800 V to the nanospray tip. Ions were introduced to the mass spectrometer, and prior to MS/MS analysis all ions except the parent ion of interest were ejected from the ion trap. MS/MS and MS³ analysis was completed using resonance excitation with the mass range up to m/z 1200 for the fragment ions.

**NMR Spectroscopy.** NMR spectra were obtained in 90 % H₂O-10 % D₂O solution at 27 °C and at a peptide concentration of 2 mM. Spectra were acquired on a Varian Inova 600 spectrometer; data matrices of 2048 detected and 512 (1024 for DQF-COSY) indirect data points with 64 (96 for ROESY) scans were recorded and processed using a 90 shifted sine bell window function (unshifted for DQF-COSY). Water signal suppression was achieved by transmitter presaturation. All experiments were performed at pH 2 (H₂O contained 0.1% TFA); under these conditions, the possibility of peptide aggregation was
reduced due to DAB-γ-amino group protonation. The assignment of $^1$H resonances was performed using standard two-dimensional DQF-COSY (28), TOCSY (29) (mixing time 70 ms), and two-dimensional NOE experiments (NOESY (30) and ROESY (31), mixing times 200 ms). The temperature coefficients of the amide proton chemical shifts were calculated using a series of one-dimensional experiments performed at four different temperatures in the range 27-42 °C. Two-dimensional transferred NOE (TRNOE) experiments (32,33) with mixing times of 200 ms were done using a mixture of mattacin and LPS that corresponded to an 8:1 w/w ratio of both components; these conditions yielded moderately broadened lines in the amide region of the mattacin 1D spectrum. A three-dimensional structure of mattacin was computed based on NOE restraints derived from the TRNOE experiment using a dynamical annealing protocol in CNS 1.1 (© Yale University). Fifty representative structures were calculated based on 62 NOEs. The NOEs were classified as strong, medium, and weak, corresponding to maximum distances of 3.0, 4.0 and 5.0 Å, respectively, based on the volumes of the assigned cross-peaks in the NOESY spectrum.

**Stereochemical Analysis of Mattacin.** Mattacin (1 mg) was hydrolyzed (1 mL 6 N HCl, 110 °C, 18 h), and the hydrolysate was dried under a nitrogen stream and then derivatized using an Alltech (Deerfield, IL) PFP-IPA (pentafluoropropyl amide – isopropyl ester) amino acid derivatization kit (#18093). The dried hydrolysate was treated with 0.2 N HCl (5 min at 110 °C) and dried under an argon stream. To this, 150 μL of acetyl chloride and 500 μL of isopropanol were added, and the mixture was heated at 110 °C for 45 min. After drying with an argon stream, the derivatizing agent, pentafluoropropyl propionic anhydride (1 mL dissolved in 2 mL of CH$_2$Cl$_2$), was added, and the solution was heated
at 115 °C for 15 min, blown dry with argon, and then solubilized in CH₂Cl₂. For standards, 10 mg each of D/L threonine, leucine, and \( \alpha,\gamma \)-diaminobutyric acid were subjected to the identical derivatization sequence. Also, 1 mg of purified polymyxin B was hydrolyzed and treated in the same fashion to serve as standard. All samples were analyzed by GC-MS under identical conditions, using a Heliflex Chirasil-Val, 50 m x 0.25 mm x 0.16 μm column (Alltech #13636), helium as carrier gas (0.6 ml/min), and a temperature gradient beginning at 90 °C (5 min hold) and ramping to 160 °C (3 °C/min) followed by a 12 minute hold.

**Isothermal Titration Calorimetry.** LPS from *E. coli* strain 055:B5 was obtained from Sigma and polymyxin B sulfate was purchased from Fluka. The polymyxin B was further purified by RP HPLC (using methods identical to those described above for mattacin) and the LPS preparation was used as purchased. Using a molecular weight estimate of 20,000 Da for the LPS monomer (34) a 0.05 mM LPS solution was prepared by dissolving LPS in 50 mM sodium phosphate buffer, pH 6.8, along with equimolar quantities of triethylamine with respect to the anionic groups in the LPS monomer (4 eq.). The solution was vortexed vigorously for 15 min and sonicated for 5 minutes prior to use. Titrations were performed using the OMEGA high-sensitivity microcalorimeter manufactured by MicroCal Inc. (Northampton, MA) as previously described (35). For measurement of heat exchanges accompanying the binding of polymyxin B or mattacin to LPS, the LPS solution was loaded into the sample cell of the calorimeter (volume = 1.4423 ml) and the reference cell was filled with water containing 0.05% sodium azide. Next, polymyxin B or mattacin, in the same buffer, was placed in a 250 μl syringe at a concentration of 1.25 mM (25-fold higher than that of the LPS). The system was allowed
to equilibrate at 20.0 °C and a stable baseline recorded before initiating an automated titration. A titration sequence involved 7.2 μl aliquot injections of polymyxin B or mattacin delivered over 10 s at 5 min intervals into the sample cell. Throughout the titration, the cell was stirred continuously at 400 rpm.
Results

Producer Strain Identification. NCBI nucleotide Blast homology search results of the 16s rRNA gene sequence revealed high homology to the *Paenibacillus* genus. Specific biochemical and morphological results, shown in Table I, revealed the bacterium to be *P. kobensis*, later named strain M.

Spectrum of Activity. Live-cell deferred inhibition assays showed that *P. kobensis* M inhibited numerous Gram-positive and Gram-negative species including among others *E. coli* O157:H7 ATCC 33150, *Salmonella* Rubislaw, and *Listeria monocytogenes*, but failed to inhibit *Pediococcus acidilactici*. Purified mattacin and polymyxin B showed the same inhibition spectrum as the live *P. kobensis* M cells with the exception that they both failed to inhibit the strains of *Listeria* and *Bacillus* tested. Furthermore, mattacin showed a consistently higher level of activity against all strains tested in this study, including activity against *Vibrio parahaemolyticus* G1-166, against which Polymyxin B was inactive. Table III shows the complete antimicrobial spectrum elucidated in this study as well as the inhibition spectrum of polymyxin B against the same organisms for comparative purposes.

Antimicrobial Peptide Production During Growth. Figure 3 shows the relationship between *P. kobensis* M growth and antimicrobial peptide production. As can be seen from this figure, production started at the exponential phase and reached its highest point during the stationary phase.

Transpositional Mutagenesis of Mattacin Biosynthetic Genes. To identify the genes related to polymyxin M production, we performed transposon insertional mutagenesis with Tn917. Transformation of *P. kobensis* M with the plasmid pLTV3 produced seven
transformants, of which one was used to produce our insertional mutagenesis library. Nine partial production mutants and one non-production mutant were obtained from a screen of approximately 7,000 colonies. PCR amplification of these mutants revealed all to contain the Tn917 transposon sequence with the wild-type producer strain lacking any endogenous transposon sequence. Plasmid sequencing from the resultant *E. coli Jm2r* transformants showed a variety of homologies to known genes as summarized in Table II.

**Mattacin Structure Elucidation.** Initial investigations into the structure of mattacin did not immediately lead to the polymyxin family of peptide antibiotics. MALDI mass spectrometric analysis suggested a molecular weight of 1157 Da (figure 4a). Standard peptide analyses were then performed consisting of Edman degradation and amino acid analysis after HCl hydrolysis. Attempts at Edman sequencing of the peptide failed, thereby indicating the presence of an N-terminal blocking moiety. Upon strong acid hydrolysis of the peptide, detectable amounts of Leu, Thr, and a third non-proteinaceous amino acid (later identified as \(\alpha,\gamma\)-diaminobutyric acid) were observed. Attempts at partial hydrolysis of the peptide with either mild base or acid failed to yield fragments amenable to Edman sequencing. With the knowledge that the peptide contained threonine, acetylation experiments were performed in the hope that the derivatized peptide might yield material suitable for x-ray crystallographic analysis. Although the acetylated peptide is not crystalline, it was determined that 8 acetyl groups were incorporated (shown by MALDI-TOF MS) indicating 8 nucleophilic moieties in the structure (figure 4b).

Extensive mass spectroscopy was done on the native peptide using MALDI-TOF tandem MS/MS techniques in an attempt to gain sequence information. This mass
spectrometry work showed the presence of a number of amino acid residues with an experimental mass of 100.064 Da having a molecular formula of C₄H₈N₂O, thereby suggesting the presence of α,γ-diaminobutyric acid moieties in the peptide. A high resolution molecular ion MH⁺ value of 1157.7370 (monoisotopic) indicated a molecular formula of C₅₁H₉₇N₁₆O₁₄. Thus it became apparent that mattacin was likely a member of the polymyxin class of antibiotics.

**NMR Spectroscopy.** Complete structure elucidation of mattacin was achieved by use of NMR spectroscopy. Initial 1D and HH-COSY experiments confirmed that mattacin belonged to the polymyxin group of antibiotics by revealing the presence of eleven amide protons and ten alpha protons (figure 5a and 5b) as well as the fatty acid side chain. The spin systems in the TOCSY spectra of mattacin were assigned to the respective residues by taking into consideration the characteristic frequencies and numbers of resonances (figure 6). Sequential assignment of the peptide is achieved using $d_{\alpha\beta}(i,i+1)$ connectivities in the ROESY spectrum as well as the connectivities determined by the HH-COSY spectrum (figure 7). A series of 1D NMR experiments at various temperatures (ranging from 27 to 42 °C) with mattacin show that two of the ring-amide protons are shielded from solvent by intramolecular H-bonding (table IV). The transferred NOE experiments performed with the mattacin/LPS mixture revealed a number of NOEs for the heptacyclic region of the peptide that were not visible prior to the addition of LPS. These NOEs (62 total) were used to produce a conformational model for comparison to that of polymyxin B (19). Matticin adopted a chair-like conformation with the sidechains of Dab 4 and 8 pointing downwards from those of Thr 6 and Leu 7 (figure 8).
Stereochemical Analysis. To verify the stereochemistry of each residue present in mattacin, a sample was hydrolyzed and the resulting amino acid mixture was derivatized to corresponding pentafluoropropyl amide - isopropyl esters for analysis using phase GC-MS. From the analysis it was clear that all 3 threonines were of L configuration (D-isomer $R_t = 10.8$ min, L-isomer $R_t = 11.2$ min) as were all 6 $\alpha,\gamma$-diaminobutyric acid residues (D-isomer $R_t = 30.6$ min, L-isomer $R_t = 31.1$ min). The leucine residue had D configuration (D-isomer $R_t = 15.5$ min, L-isomer $R_t = 16.3$ min). A sample of polymyxin B, derivatized and analyzed in the same manner (to serve as a secondary standard), gave results in complete agreement with the known stereochemistry for each residue. Thus the sequence, constituent amino acids, lipid portion, and connectivity of mattacin were identical to the structure proposed earlier by Russian workers for polymyxin M (13, 14, 15, 16), although no conformational information or detailed comparison to polymyxin B were reported.

Isothermal Titration Calorimetry. To investigate whether mattacin behaved in a fashion similar to that ascribed to polymyxin B in its binding to LPS (31), a calorimetric investigation was performed. Both peptides where titrated into a solution of LPS and the heats of binding monitored. The results of these binding assays were complex. However, it was evident that both peptides bound LPS in almost identical fashion. The binding isotherms were virtually indistinguishable (figure 9) and suggested that both peptides were interacting with the same receptor site(s) present in LPS. The results did not support simple 1:1 interaction, but rather a model with a high probability of identical stoichiometry and a series of sequential binding events.
Discussion

In recent years a large number of antimicrobial peptides from Gram-positive bacteria have been discovered, including ribosomally produced bacteriocins (36,37,38). The non-ribosomally generated polymyxins represent one of the earliest classes of structurally unique peptide antibiotics to be identified (17). *Paenibacillus* spp. are Gram-positive, spore-forming bacteria from which polymyxins have been isolated and their *in vitro* biosynthesis by cell-free enzyme systems has been successfully demonstrated (39). Although the first member of the polymyxin family, polymyxin B, was discovered in 1947 (17), the genetic control of their biosynthesis has not been described. We utilized the Tn917 transposon to study the mattacin (polymyxin M) biosynthesis genes and screened approximately 7,000 colonies for production mutants.

The majority of the production mutants exhibited decreased levels of production as opposed to a complete loss of production. This is not consistent with other studies using Tn917 for iturin and fengycin biosynthesis genes (40,41). In those studies, the transposon disrupted the NRPS genes and blocked the antimicrobial peptide synthesis completely. The identified genes in our study show low homology to other known genes in the NCBI database. The biosynthesis genes of mattacin in *Paenibacillus* spp. may be quite different from other peptide synthases in DNA sequence, which may be the result of different evolutionary gene origin. Despite all the differences, our study did show some similarities between mattacin biosynthesis and the synthetic processes of other peptide antibiotics, including the use of non-ribosomal peptide synthetases for peptide growth and ABC transporters for secretion.
Structural analysis of mattacin utilized a combination of chemical degradations, mass spectrometry, and multidimensional NMR analyses to obtain primary sequence, identity of the lipid side chain, connectivity, stereochemistry, and conformational preferences. As is common for small peptides, mattacin shows considerable conformational flexibility when pure in solution, as shown by the absence of extensive long range NOE interactions. Recent NMR investigations by Kidric and Pristovsek (19) have shown that in solution, two of the ring-amide protons in polymyxin B participate in intramolecular hydrogen bonding. This is detected by the temperature dependencies of the amide proton chemical shifts – specifically, amide protons that are least affected by temperature change are likely shielded from solvent by participation in H-bonding. In mattacin, it was observed that the amide protons of DAB 8 and the side chain of DAB 4 are both shielded, as in polymyxin B. These results suggest that mattacin and polymyxin B, though containing structural differences, are both highly flexible in solution and can adopt similar conformations.

It has long been believed that polymyxins elicit their bactericidal effects by binding to and disrupting the action of lipopolysaccharide (LPS) – the major antigen of the outer membrane of Gram-negative bacteria (42). LPS contains 3 major structural components, Lipid A, a core oligosaccharide, and an outer polysaccharide composed of repeating hetero-oligosaccharide subunits. Lipid A is a hydrophobic, lipid-rich moiety that harbors the endotoxic principle of LPS and is the most highly conserved part of the structure, containing two glucosamines, two phosphate esters, and six fatty acid chains (43). The proposed binding model for the polymyxin-LPS conjugate involves ionic interactions between the side chain amino groups of the polymyxin peptide cycle.
(positively charged in acidic medium) and the negatively charged phosphate groups of the lipid A disaccharide (44). Also proposed to contribute to binding is the hydrophobic interaction between the nine carbon fatty acid side chain of the polymyxin and the fatty acid portion of lipid A (figure 10).

Using transferred NOE 2D NMR techniques, Kidric and Pristovsek (19) recently investigated the preferred conformation(s) induced in polymyxin B when in solution with LPS from *E. coli*. Their results indicate that although the peptide is highly flexible, the side chain of Dab 8 and the γ-amide NH of Dab 4 as well as the amide NH of Dab 8 with the side chain of Dab 4 are in close proximity while bound to LPS. In performing these experiments with mattacin and the same LPS preparation, however, we did not detect the same correlations. Our conformational model in fact is somewhat different than that of polymyxin B (figure 8). While the side chains of all three DAB residues contained in the heptacycle were on the opposite side of the molecule from the hydrophobic Phe and Leu side chains in the polymyxin B structure, the bend in the mattacin heptacycle was in the opposite direction. This resulted in a less dramatic separation of hydrophobic and hydrophilic sidechains. Whether this was due to our experiments not being able to detect the critical NOEs seen for polymyxin B, or whether the conformational differences have an explanation owing to the structural differences is not clear. Mattacin was significantly different at residues 6 and 7 (D-Leu, L-Thr) compared with the corresponding residues in polymyxin B (D-Phe, L-Leu). It was in this region of the heptacycle that the greatest conformational variation in the two models was observed, and it seems reasonable to suggest that the significantly diverse hydrophobic/hydrophilic residues present in the two peptides contribute to these differences.
To ascertain whether maccatin behaved in a manner similar to polymyxin B in its interaction with LPS, isothermal titration calorimetry (ITC) was employed. The thermodynamics of polymyxin B binding to LPS have been previously investigated using ITC by the group of Surolia (34). In these studies, a highly processed LPS preparation from *E. coli* was used (extensive treatments with proteases, chelating agents, and purifications via dialysis and size exclusion chromatography). Using these conditions, which lead to smaller fragments of LPS, Surolia obtained results supporting a simple 1:1 binding between polymyxin B and LPS. We chose to use an LPS from the same *E. coli* strain, but without extensive processing because the primary interest was the comparison of maccatin with polymyxin B and their respective binding to LPS. The same stock LPS solution and concentrations were used for ITC experiments with both maccatin and polymyxin B. As seen in figure 9, the binding isotherms for both peptide titrations were almost indistinguishable. The data does not support a simple binding model and the ORIGIN analysis software could only fit the data using a complex sequential binding model. Initial interaction of the polymyxins with unprocessed LPS appears to lead to disruption of tertiary structural arrangements, thereby exposing additional lipid A binding sites. The simple 1:1 binding of polymyxin B seen in previous experiments (34) with highly processed lipid A may be due to the predominance of smaller unconglomerated units. Whether this form provides a more accurate model of what occurs with living bacterial cells remains uncertain. However, our results clearly suggest that maccatin binds to LPS in a similar manner as polymyxin B.

Results from the activity assays for both maccatin and polymyxin B showed that the two peptides have virtually indistinguishable spectra of activity. Maccatin did appear
to be slightly more active in most cases, but these differences are not very significant (less than an order of magnitude). An interesting observation in the activity assays was that all strains of *Listeria* and *Bacillus* were inhibited by the live cells of *P. kobensis* M, but the purified polymyxins B and M had no effect. This suggests that another compound(s) is produced by this organism which is either lethal to *Listeria* and *Bacillus* on its own, or acts in synergy with another compound(s), possibly the polymyxin, to elicit its killing effects. Many multiple-component bacteriocin systems are now known to be produced by Gram-positive organisms and have been recently reviewed (11,45). Future investigations aimed at determining whether *P. kobensis* M produces other novel antimicrobial compounds and at elucidating the details of polymyxin biosynthesis are in progress.

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Figure Legends

Figure 1. General structure of polymyxins

Figure 2. Structure of mattacin (polymyxin M)

Figure 3. Mattacin production during *P. kobensis* M growth

Figure 4a. MALDI mass spectrum of mattacin

Figure 4b. MALDI mass spectrum of octa-acetylated mattacin

Figure 5a. 1D HNMR spectrum of mattacin with expansion of amide region

Figure 5b. HH COSY identifying α-H/amide-H correlations

Figure 6. TOCSY spectrum identifying spin system for all ten residues

Figure 7. Connectivities observed in ROESY and HH-COSY spectra leading to sequence assignment in mattacin

Figure 8. Conformational model of mattacin heptacycle based on tNOE data obtained from mattacin/LPS mixture

Figure 9. Binding isotherms resulting from the titration of LPS with both mattacin and polymyxin B

Figure 10. Schematic representation of a generic polymyxin molecule (oval with tail) bound to lipid A
### TABLE I

**Chemical and Morphological Characteristics of the Producer Strain**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spore Shape</td>
<td>Oval</td>
</tr>
<tr>
<td>Vegetative Cell Volume</td>
<td>5 µm x 1 µm</td>
</tr>
<tr>
<td>Resistance to polymyxin</td>
<td>No</td>
</tr>
<tr>
<td>Growth at 50 °C</td>
<td>No</td>
</tr>
<tr>
<td>Growth in presence of .001% lysozyme</td>
<td>Yes</td>
</tr>
<tr>
<td>Growth in presence of 5% NaCl</td>
<td>No</td>
</tr>
<tr>
<td>Growth at pH 6</td>
<td>Yes</td>
</tr>
</tbody>
</table>

### TABLE II

**NCBI Blast Homology Search Results of Tn917 Flanking Fragments**

<table>
<thead>
<tr>
<th>Plasmid No.</th>
<th>Mutant type</th>
<th>Homologous gene from LacZ direction</th>
<th>Homologous gene from MCS direction</th>
</tr>
</thead>
<tbody>
<tr>
<td>33-1</td>
<td>Lower-level production</td>
<td>Vector sequence</td>
<td>Response regulator-like gene and vector sequence</td>
</tr>
<tr>
<td>33-2</td>
<td>Lower-level production</td>
<td>Peptide antibiotic synthetase gene</td>
<td>ABC transporter gene in tyrocidine biosynthesis operon</td>
</tr>
<tr>
<td>34</td>
<td>Lower-level production</td>
<td>Vector sequence</td>
<td>Acyl-CoA synthase</td>
</tr>
<tr>
<td>55</td>
<td>Lower-level production</td>
<td>Peptide antibiotic synthetase gene</td>
<td>ABC transporter gene in tyrocidine biosynthesis operon</td>
</tr>
<tr>
<td>56</td>
<td>Lower-level production</td>
<td>Peptide antibiotic synthetase gene</td>
<td>ABC transporter gene in tyrocidine biosynthesis operon</td>
</tr>
<tr>
<td>239</td>
<td>Lower-level production</td>
<td>Probable transcriptional regulator</td>
<td>Probable transcriptional regulator</td>
</tr>
<tr>
<td>301</td>
<td>Non-production</td>
<td>No significant alignment</td>
<td>Sensor histidine kinase in two-component regulator system</td>
</tr>
<tr>
<td>410</td>
<td>Lower-level production</td>
<td>ABC Transporter</td>
<td>Transcription Regulator</td>
</tr>
<tr>
<td>411</td>
<td>Lower-level production</td>
<td>ABC Transporter</td>
<td>16s rRNA gene</td>
</tr>
<tr>
<td>460</td>
<td>Non-production</td>
<td>Peptide antibiotic synthetase gene</td>
<td>Peptide antibiotic synthetase gene</td>
</tr>
</tbody>
</table>
TABLE III
Antimicrobial spectrum of live *P. kobensis* M and purified Peptide Antibiotic

<table>
<thead>
<tr>
<th>Indicator Strain</th>
<th>Live <em>P. kobensis</em> M</th>
<th>MIC (μM)</th>
<th>Mattacin</th>
<th>Polymyxin B</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em> BF2</td>
<td>+</td>
<td>3</td>
<td>3</td>
<td>25</td>
</tr>
<tr>
<td><em>Escherichia coli</em> O157:H7 380-94</td>
<td>+</td>
<td>6</td>
<td>6</td>
<td>25</td>
</tr>
<tr>
<td><em>Escherichia coli</em> O157:H7 933</td>
<td>+</td>
<td>12.5</td>
<td>12.5</td>
<td>25</td>
</tr>
<tr>
<td><em>Escherichia coli</em> O157:H7 3081</td>
<td>+</td>
<td>12.5</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em> O157:H7 ATCC 33150</td>
<td>+</td>
<td>12.5</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em> ATCC 25922</td>
<td>+</td>
<td>6</td>
<td>12.5</td>
<td></td>
</tr>
<tr>
<td><em>Yersinia enterocolitica</em></td>
<td>+</td>
<td>3</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td><em>Klebsiella pneumonia</em></td>
<td>+</td>
<td>12.5</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td><em>Shigella soneii</em></td>
<td>+</td>
<td>3</td>
<td>12.5</td>
<td></td>
</tr>
<tr>
<td><em>Alcaligenes faecalis</em> ATCC 8750</td>
<td>+</td>
<td>12.5</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas putida</em> D3375</td>
<td>+</td>
<td>12.5</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em></td>
<td>+</td>
<td>12.5</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>+</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td><em>Salmonella Typhimium</em> 14028</td>
<td>+</td>
<td>12.5</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td><em>Salmonella Rubislaw</em> F2833</td>
<td>+</td>
<td>25</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td><em>Salmonella Gaminara</em> 140665</td>
<td>+</td>
<td>25</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td><em>Acinetobacter baumanii</em></td>
<td>+</td>
<td>12.5</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td><em>Vibrio parahaemolyticus</em> G1-166</td>
<td>+</td>
<td>12.5</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>Vibrio parahaemolyticus</em> G1-172</td>
<td>+</td>
<td>12.5</td>
<td>25</td>
<td></td>
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<tr>
<td><em>Alicyclobacillus acidoterrestris</em> VF</td>
<td>+</td>
<td>50</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em> 2289</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>*</td>
</tr>
<tr>
<td><em>Listeria innocua</em></td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>*</td>
</tr>
<tr>
<td><em>Listeria ivanovii</em></td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>*</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em> ATCC 6633</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>*</td>
</tr>
<tr>
<td><em>Bacillus cereus</em></td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>*</td>
</tr>
<tr>
<td><em>Pediococcus acidlactici</em></td>
<td>-</td>
<td>NT</td>
<td>NT</td>
<td></td>
</tr>
</tbody>
</table>

+: inhibition, -: no inhibition, NT = not tested, *: no inhibition up to 100 μM
<table>
<thead>
<tr>
<th>residue</th>
<th>Temperature coefficient $(10^{-3}$ ppm/K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dab1</td>
<td>-7.8</td>
</tr>
<tr>
<td>Thr 2</td>
<td>-5.3</td>
</tr>
<tr>
<td>Dab 3</td>
<td>-6.5</td>
</tr>
<tr>
<td>Dab 4</td>
<td>-5.6</td>
</tr>
<tr>
<td>Dab 4γ</td>
<td><strong>-3.9</strong></td>
</tr>
<tr>
<td>Dab 5</td>
<td>-4.8</td>
</tr>
<tr>
<td>Leu 6</td>
<td>-5.6</td>
</tr>
<tr>
<td>Thr 7</td>
<td>-7.1</td>
</tr>
<tr>
<td>Dab 8</td>
<td><strong>-2.6</strong></td>
</tr>
<tr>
<td>Dab 9</td>
<td>-4.7</td>
</tr>
<tr>
<td>Thr 10</td>
<td>-5.5</td>
</tr>
</tbody>
</table>

*Coefficients marked in bold indicate amides protected from solvent.*
Figure 1.

\[ \text{L-DAB = H}_2\text{N} \begin{array}{c} O \text{H} \\
\text{NH}_2 \end{array} \]

\[ \text{C}_8 = \begin{array}{c} - \text{H} \\
\text{C}_8 \end{array} \quad \text{or} \quad \begin{array}{c} - \text{H} \\
\text{C}_8 \end{array} \]

A = D-Leu or D-Phe
B = L-Thr, L-Leu, L-Phe, or L-Ileu
Figure 2.
Figure 3.
Figure 4b.
Figure 5a.
Figure 5b.
Figure 7.
Figure 8.
Figure 9.

Mattacin

Polymyxin B

Molar Ratio

Time (min)

μcal/sec

kcal/mole of injectant

Molar Ratio

Time (min)

μcal/sec

kcal/mole of injectant
Figure 10.
References

Isolation, structural characterization, and properties of mattacin (polymyxin M), a cyclic peptide antibiotic produced by Paenibacillus kobensis M
Nathaniel I. Martin, Haijing Hu, Matthew M. Moake, John J. Churey, Randy Whittal, Randy W. Worobo and John C. Vederas

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