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Biosynthesis of isotopically labeled gramicidins and tyrocidins by *Bacillus brevis*

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Abstract

The three-dimensional structure of bilayer-associated gramicidin A is available from a structural data base. This and related peptides are, therefore, ideal model compounds to use during the implementation and development of new NMR techniques for the structural investigations of membrane proteins. As these methods rely on the isotopic labelling of single, selected or all sites, we have, investigated and optimised biochemical protocols using different strains of the Gram-positive bacterium *Bacillus brevis*. With newly developed schemes for isotopic labelling large amounts of gramicidin and tyrocidin enriched with stable isotopes such as ^{15}N or ^{15}N/^{13}C have been obtained at low cost. A variety of analytical and spectroscopic techniques, including HPLC, mass spectrometry and NMR spectroscopy are used to characterise the resulting products.

Abbreviations: DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; HPLC, high performance liquid chromatography; MALDI MS, matrix-assisted laser desorption mass spectrometry; NMR, nuclear magnetic resonance.

Introduction

Despite their widespread occurrence little is known about the conformational details of membrane proteins. This is mostly due to the technical difficulties encountered during the expression and biochemical purification of this class of proteins in quantities that are sufficient for structural studies. In addition, even if these problems can be overcome, in many cases classical solution NMR and diffraction techniques still fail due to the large size of membrane proteins and their resistance to form ordered arrays in native environments. As a consequence, only a few high-resolution structures of membrane proteins have been obtained to date (e.g., (Toyoshima et al., 2000; Garavito, 1998; Tsukihara and Lee, 1999; Palczewski et al., 2000).

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Instead solid-state NMR spectroscopy has been used to answer specific questions about the structure, dynamics and organisation of membrane proteins. This technique requires the provision of samples labelled with stable isotopes at specific, selective or all sites (Cross, 1997; Watts, 1998; Davis and Auger, 1999; Bechinger et al., 1999; Bechinger, 2000). Further development of solid-state NMR techniques for membrane protein structural investigations will depend on the availability of small polypeptides that can be reconstituted into oriented or non-oriented lipid bilayers at high concentrations. These model compounds should be well characterized so that experiments can be designed and analysed in a quantitative manner. Evidently the availability of simple but uniformly labelled polypeptide samples during the development of new solid-state NMR methods is particularly desirable.

The membrane-associated structure of the backbone and side chains of gramicidin A, a pentadecameric peptide, has been solved at high resolution us-

ing orientational constraints which were derived from solid-state NMR spectra (reviewed in Cross, 1997; Davis and Auger, 1999; Bechinger, 1999). In order to perform these studies peptides have been prepared using solid-phase peptide synthesis (Fields et al., 1989), labelled with stable isotopes at one or a few selected sites, purified and reconstituted into oriented phospholipid bilayers at high peptide-to-lipid ratios. Due to the decreasing efficiency during solid-phase peptide synthesis, which is encountered when large numbers of hydrophobic or bulky amino acids are to be added to the growing peptide chain, this procedure is at present not applicable to large membrane proteins. Furthermore, if more extensive or multiple labelling strategies are required the chemical approach quickly becomes laborious and costly. Due to its small size, its known structure and high stability also in membrane environments gramicidin A labelled with ^{15}N, ^{13}C and/or ^{2}H would provide an ideal model system for the development of new solid-state NMR methods. Therefore, in the present study we have investigated the possibilities of labelling this peptide with ^{15}N or $^{15}N/^{13}C$ uniformly or selectively by biochemical methods. For gramicidin A to be useful as a model system the biochemical preparation of this peptide should be easy and cost-efficient.

Gramicidin is produced by the Gram-positive bacterium *Bacillus brevis* (strain ATCC 8185). The ethanol extract of the Bacillus is referred to as tyrothricin and is produced in large amounts during sporulation (up to 500 mg l^{-1}) (Dubos and Hotchkiss, 1941; Stokes and Woodward, 1943). Whereas the major component (80%) of this extract is the circular decapeptide tyrocidin, the peptide antibiotic gramicidin constitutes a second component, to the extent of 10–15%. Gramicidin has been studied in the past using a wide variety of techniques including X-ray, solution and solid-state NMR spectroscopy and is also used in technical applications such as biosensors (Cornell et al., 1999). The unlabelled peptide is commercially available at a low cost.

The sequence of gramicidin A, which constitutes the major component of gramicidin (85% abundance), is:

HOC-V-G-A-L-A-V-V-V-W-L-W-L-W-L-W-NH-CH_2CH_2OH.

In the above sequence the D-amino acids are underlined. The peptide is uncharged due to the absence of acidic or basic residues. In addition, a formyl group blocks the N-terminus and an ethanolamine group is attached to the C-terminus. It has been shown that the four tryptophans at the C-terminus are important for channel activity. Five analogues of gramicidin A are also available from the bacterial extracts. Whereas gramicidin B differs from gramicidin A by a single $Trp_{11} \rightarrow$ Phe substitution, gramicidin C exhibits a $Trp_{11} \rightarrow$ Tyr replacement. In addition, a $Val_1 \rightarrow$ Ile modification exists for each of these analogues (Killian, 1992).

Gramicidin incorporates readily in phospholipid membranes as a right-handed $\beta^{6.5}$ helix (Cross, 1997). The intra-molecular hydrogen-bonding pattern of this structure is similar to that found in anti-parallel β-sheets. The resulting helix is composed of 6.5 amino acids per turn. Because of the alternating D- and L-amino acids, the hydrophobic side chains are directed towards the outside of the helix whereas the hydrophilic backbone lines the inside of the resulting pore structure. The $\beta^{6.5}$ helix of gramicidin spans half the thickness of a bilayer. Two peptides associating from opposing membrane layers can form a membrane-spanning cation-selective pore, thereby developing antibiotic activity against Gram-positive bacteria.

The circular tyrocidin, which is the major component of the *B. brevis* ethanol extract, is less well characterized. The sequence of tyrocidin A is (King and Craig, 1955):

-V-O-L-F-P-F-F-N-Q-Y-.

Again the D-amino acids are underlined and O represents ornithine. Consecutive single amino acid substitutions of tyrocidin result in the A, B and C analogues. Whereas a $Phe_6 \rightarrow$ Trp substitution characterises tyrocidin B, an additional $Phe_7 \rightarrow$ Trp substitution is present in tyrocidin C. Other amino acid substitutions include a $Tyr_{10} \rightarrow$ Trp or Phe substitutions which are observed for all analogues (Battersby and Craig, 1952; King and Craig, 1955). Tyrocidin is active against Gram-negative bacteria and is used in a wide variety of pharmaceutical compositions such as dental care products, mouth waters, cough medicines and eye-drops.

In early reports the strain BG (ATCC 8185) was used to produce tyrothricin (Dubos and Hotchkiss, 1941). Our attempts to use the strain BG for the production of gramicidin in a medium obtained from *E. coli* extracts were unsuccessful (Table 1). Furthermore, it seems that the stringent requirements for the biosynthesis of tyrothricin by this strain make the de-

Table 1. Optical denisty at 600 nm (cell cultures) and 280 nm (ethanol extracts) of *B. brevis* strains DSM-362 and DSM-5818 when grown on different media

	DSM-362			DSM-5618		
	OD_{600}	OD_{280}	Gramicidin[a]	OD_{600}	OD_{280}	Gramicidin[a]
LB	1460	411	–	4710	1148	+
Peptone	3240	525	–	5100	1272	+++
Celtone	130	64	NA	4510	1217	+++
Martek-9	2150	111	–	3910	126	+
All-AA	820	514	NA	2430	1059	NA
NH_4Cl	1860	207	–	1400	178	–
DL-Leu	4	3	–	18	5	NA
DL-Leu + Pipecolic acid	4	3	–	5	5	NA
DL-Pipecolic acid	4	3	–	50	5	–

[a]The HPLC peak intensities recorded at 280 nm at elution times of gramicidins were compared to those of all tyrothricins and classified in the following manner: <0.15: −; 0.15–0.25: +; 0.4–0.6: +++, NA: not analyzed.

sign of efficient isotopic labelling techniques difficult, if not impossible. However at least two other *Bacillus brevis* strains, namely ATCC 10068 (Mach et al., 1963; Nicholson et al., 1987) and Culture-TC (Dubos and Hotchkiss, 1941), were reported to produce tyrothricin or compounds of similar physical characteristics. Remarkably, the phenotype of these two strains is very different from that of strain BG.

In the present report we describe investigations of Culture-TC grown on media of different composition and compare the results with those obtained with strain BG. It should be noted that the synthesis of gramicidin is not directly DNA-encoded but regulated by a multi-enzyme complex. Therefore, considerable attention will be given to determine the physical characteristics of the resulting products of the ethanol extract. We will show that the gramicidins and tyrocidins produced by Culture-TC are identical to those produced by strain BG, albeit with different ratios of gramicidin and tyrocidin analogues. The isotopic labelling of tyrotricin components using Culture-TC will be described, thereby offering alternative and up-to-date methods to a previously published protocol (Nicholson et al., 1987).

Materials and methods

Strains

Bacillus brevis strains DSMZ-362 (ATCC-8185) and DSMZ-5618 were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany.

Media

The commercially available media Martek-9 and Celtone are from Martek Biosciences Corp. (Columbia, USA) and were used without modification. All amino-acids, DL-pipecolic acid and tyrothricin are from Sigma (Deisenhofen, Germany). Peptone and yeast extract are from Life Technologies (Paisley, Scotland). All other chemicals are of analytical grade from various sources. The composition of the media is as follows. One litre of base medium contains: 11.3 g Na_2HPO_4, 3.5 g NaH_2PO_4 (pH 7.4), 0.5 g NaCl, 10 g glucose, $MgSO_4$ (0.5 mmol), $MnSO_4$ (1.5 μmol), $FeSO_4$ (2 μmol), $CuSO_4$ (0.2 μmol), $FeCl_3$ (0.75 μmol), KI (0.1 μmol), $CaCl_2$ (0.1 mmol), thiamin (10 nmol), folic acid (1 nmol), boric acid (1 nmol). The base medium is supplemented with either of the following: (1) 10 g peptone, (2) 10 g peptone + 5 g yeast extract, (3) 2.5 g DL-leucine, (4) 2.5 g DL-leucine + 0.2 g DL-pipecolic acid, (5) a mixture of all common L-amino acids (0.1 g each) except proline which was replaced by 0.2 g DL-pipecolic acid, (6) NH_4Cl and (7) 2.5 g DL-pipecolic acid.

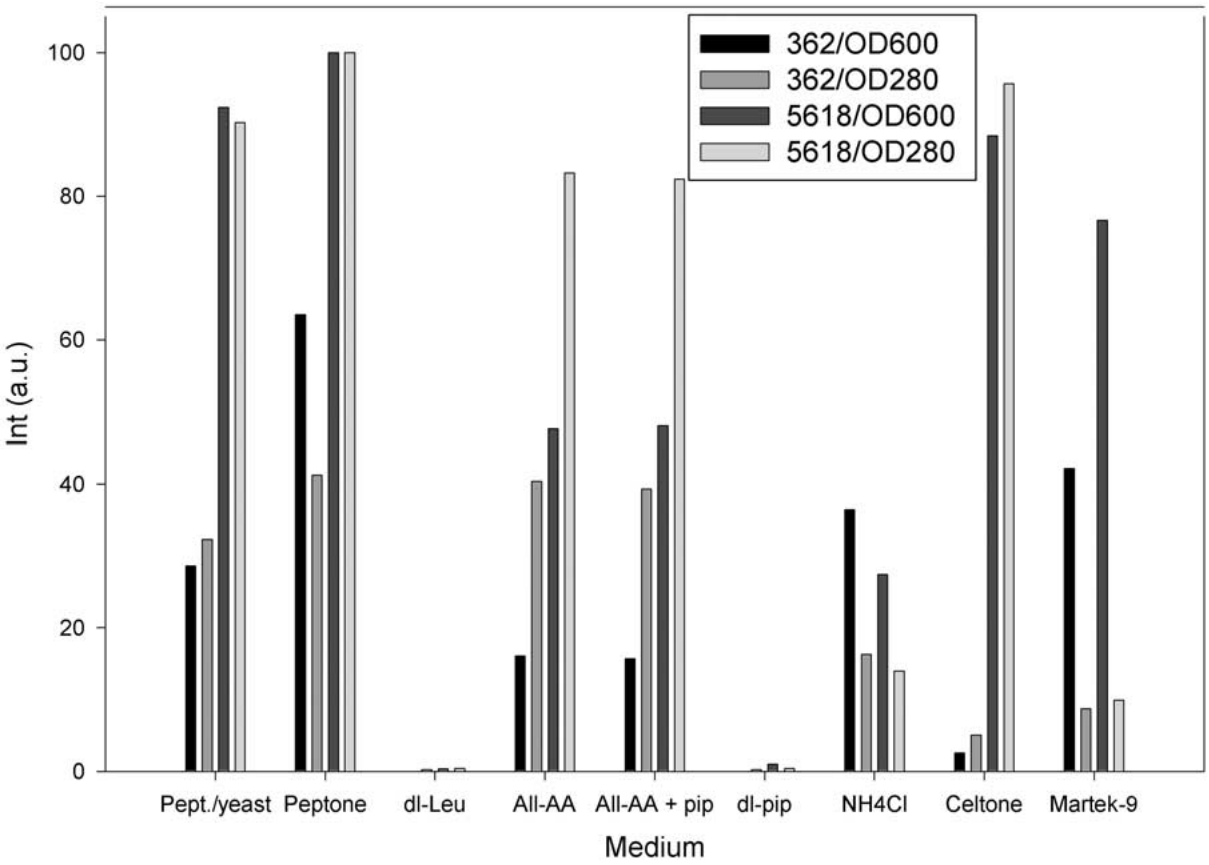

Figure 1. Bar diagram of the optical densities obtained in different fermentation media of *Bacillus brevis* cultures incubated for 7 days at 37 °C (OD_{600}) and of their respective ethanol extracts (OD_{280}). Strains DSM-362 and DSM-5618 were used. Intensities are given in arbitrary units.

Yeast extract was prepared by growing yeast on a minimal medium with NH_4Cl as the only nitrogen source. The cells were harvested by centrifugation, the resulting pellet re-suspended in double-distilled water and autoclaved. Aliquots of the resulting emulsion were crushed in a mortar filled with glass beads and the resulting fluid was collected.

Peptone medium was prepared by growing *E. coli* on a minimal medium with NH_4Cl as the only nitrogen source. After harvesting, the cells were resuspended in a phosphate buffer and pancreatin as well as DNAase were added. The cells were broken by passage through a French press and incubated for 7 days at 37 °C. The digestion was stopped by autoclavation. The last step removes the stench that accompanies digestion with pancreatin. The peptone/yeast medium was prepared by mixing the yield of 5 litre fermentations of yeast and *E. coli* at a 1:1 ratio to obtain 1 litre of medium.

Growth conditions

To produce spores *Bacillus brevis* is grown by shaking (200 rpm) for three days at 37 °C in a base medium containing 2.5 g l^{-1} asparagine. Thereafter the culture is heated to 70 °C for 20 min, which kills all living bacteria leaving only the spores to survive. 200 µl of this culture is used to start the main culture (100 ml in a 500 ml Erlenmeyer flask). The main culture is stored at 37 °C for 7 days without shaking or active aeration. Two percent (v/v) of a 100% (w/v) trichloric acid solution is added and the culture is left to stand for 4 h at 4 °C. The cells are harvested by centrifugation and re-suspended in 25 ml ethanol/100 ml of culture. The ethanol suspension is sonicated at 4 °C for 10 min with a 50% duty cycle. The optical density at 280 nm is used as a measure for tyrothricin production. Free amino acids are removed from the ethanol extract by passage through a column of acid alumina. Thereafter the extract is concentrated to a volume of 5 ml by rotary evaporation and injected in 200 ml of 200 mM

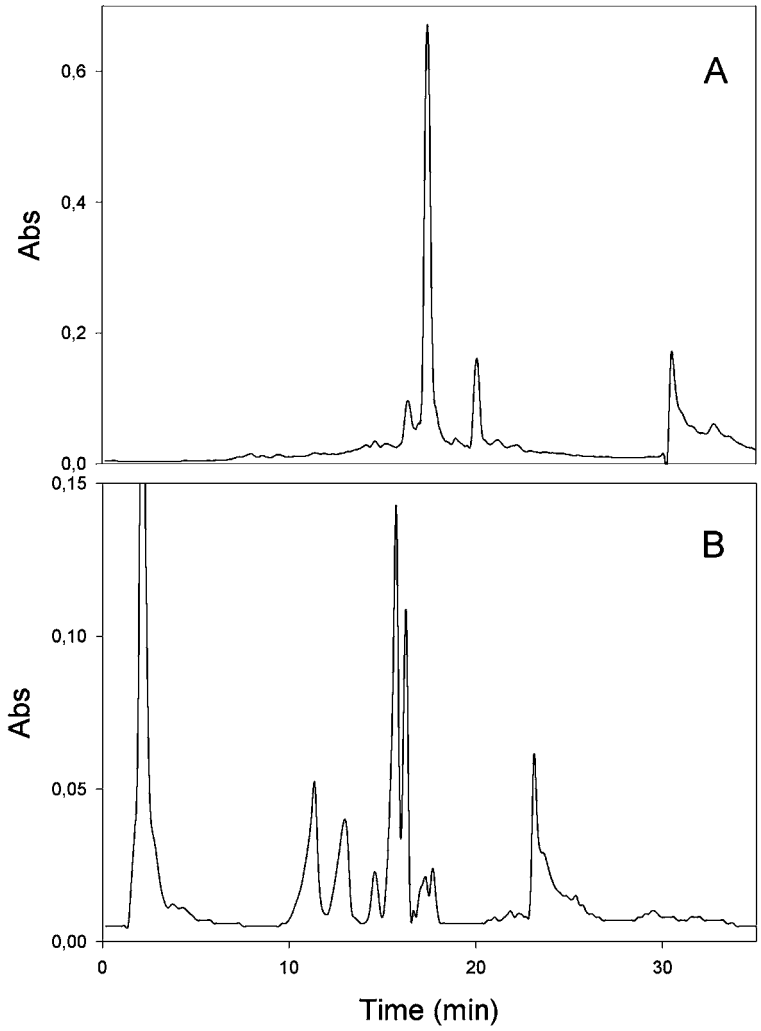

Figure 2. HPLC separation of ethanol extracts from *B. brevis (TC)* cultures. A. Gramicidins were separated using a gradient from 250/10/1 to 150/10/1 ($CHCl_3/CH_3OH/H_20$, v/v/v) and a column packed with Nucleosil at a flow rate of 8 ml min^{-1}. The main peak is that of Val1-gramicidin A. B. HPLC separation of tyrocidins was performed on a reversed phase C-18 Nucleosil column with a gradient of 70% methanol in water to 100% methanol at 0.8 ml min^{-1}. The detection wavelength was 280 nm.

NaCl. Tyrothricin precipitates as a white powder and is separated in pure form by centrifugation. The pellet is then dissolved in $CHCl_3/CH_3OH/H_2O$ (100/10/1 = v/v/v). Growing strain TC in celtone medium yields about 80 mg of gramicidin and 300 mg of tyrocidin per litre. After purification 50 mg of gramicidin A are obtained.

HPLC

Analytical HPLC separation was performed on a reversed phase C-18 column (ET 250/4 Nucleosil 100-5 C-18, Macherey and Nagel, Düren, Germany) with a gradient of 70% methanol in water to 100% methanol in 20 min and a detection wavelength of 280 nm at 0.8 ml min^{-1}. Preparative HPLC was performed using a gradient from 250/10/1 to 150/10/1 ($CHCl_3/CH_3OH/H_20$ = v/v/v) in 30 min using a column packed with Nucleosil (SP250/21 Nuc. 100-7u Macherey and Nagel, Düren, Germany) and a flow rate of 8 ml min^{-1}. In a previous study MALDI-MS was used to determine the purity and identity of the individual fractions (Kinder, 1999). The peptides elute in the following order (cf. Figure 2A): Ile_1-gB, Val_1-gB (both in minor amounts), Ile_1-gA (peak at 16′), Val_1-gA (main peak at 17′), Ile_1-gC (18.5′, small peak) and Val_1-gC (20′, major peak). Alternatively, in analogy

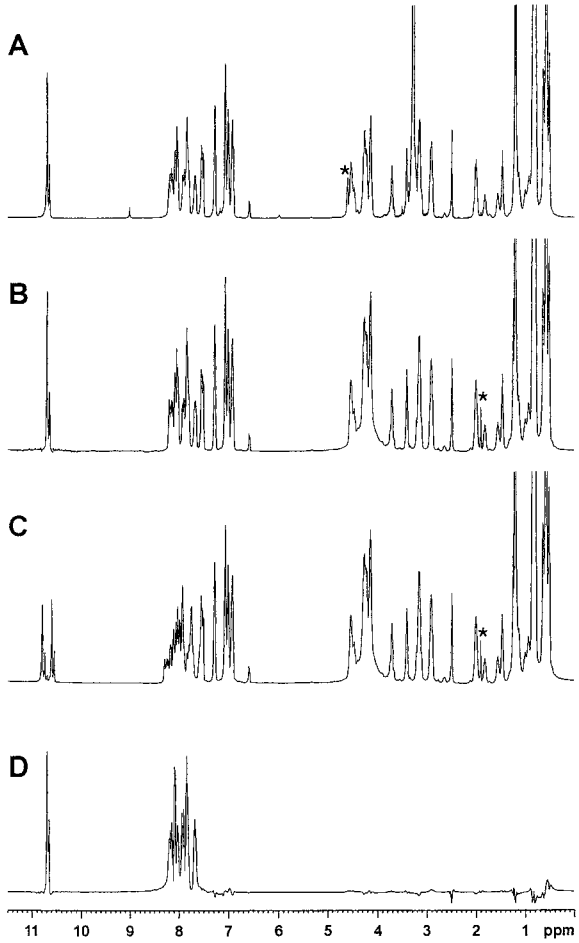

Figure 3. NMR spectra of gramicidin A preparations in DMSO-d6. (A) Commercial product (not labelled). (B and C) ^{1}H spectra of ^{15}N labelled Val1-gramicidin A from culture TC. The main peak collected from the preparation shown in Figure 2A has been used for these NMR studies. (B) ^{15}N decoupled. (C) In the presence of ^{1}H-^{15}N couplings. (D) One-dimensional ^{1}H-^{15}N correlation experiment. Only protons that exhibit heteronuclear *J*-coupling in the vicinity of 95 Hz exhibit their full intensity. Detailed resonance assignments are published in (Hawkes et al., 1987).

to the analytical separation, a gradient of 70 to 100% methanol in water is used on a reversed phase C-4 column (SP 250/21 Nucleosil 300-7 C4, Macherey and Nagel, Düren, Germany) at a flow rate of 8 ml min^{-1}.

Mass spectrometry

For mass determination matrix-assisted laser desorption mass spectrometry (MALDI MS) was applied using a Bruker Reflex III mass spectrometer.

Solution NMR spectroscopy

Gramicidin fractions are dissolved in DMSO-d_6 at a concentration of approximately 20 mg ml^{-1}. ^{1}H spectra are recorded on a Bruker Avance NMR spectrometer operating at a proton frequency of 500 MHz with and without broadband ^{15}N MLEV decoupling. ^{1}H-detected heteronuclear ^{1}H-^{15}N correlation spectra were recorded using a Δ delay of 5.2 ms (Bax et al., 1983).

Solid-state NMR spectroscopy

In order to prepare oriented membrane samples, 2 μmol of purified peptide was co-dissolved with 60 μmol of DMPC in 300 μl $CHCl_3$. 10 μl was applied to each glass cover slip (12 × 4 mm) and dried under a stream of nitrogen gas. Exposure of the sample to high vacuum over night ensures removal of the remaining solvent. The plates were then stacked inside a glass cuvette (outer dimensions of 16 × 6.6 × 6.6 mm), 50 μl water was added and the cuvette was tightly sealed with a glass lid and epoxy glue. The cuvette was stored at 37 °C. The static oriented samples were investigated at 9.34 Tesla using a Bruker Avance spectrometer. To ensure a good filling factor, either modified commercial or home-built NMR probes were used (Bechinger and Opella, 1991).

Solid-state ^{31}P NMR spectra of the phospholipid membranes were recorded at 310 K using a pulse echo sequence (Rance and Byrd, 1983) with the following experimental parameters: Spectral width 30 kHz, ^{1}H field strength ∼20 kHz, ^{31}P field strength ∼50 kHz, acquisition time 8 ms, echo delay 40–60 μs, and a recycle delay of 5 s.

Solid-state ^{15}N NMR spectra were recorded at 310 K using cross polarisation followed by a Hahn echo (Pines et al., 1973). The following parameters were used: sweep width 30 kHz, ^{1}H and ^{15}N field strength ∼20 kHz, contact pulse 0.6-1 ms, acquisition time 4.3 ms, echo delay 40–60 μs and recycling delay 2–3 s. Exponential line broadening (100 Hz) was applied before Fourier transformation. The ^{15}N ppm scale is relative to a powder of NH_4Cl (41.5 ppm). For PISEMA experiments the ^{1}H and ^{15}N field strengths were increased to 63 and 50 kHz, respectively (Ramamoorthy et al., 1999). The acquisition time in t_2 was 8.5 ms. The t_1 domain was incremented by 3π-2π phase and frequency-switched Lee Goldburg spin lock pulses, resulting in a dwell time of 34.66 μs (Lee and Goldburg, 1965; Bielecki et al., 1990). To avoid the sample from heating excessively, the total length of t_1

spin lock pulses was limited by terminating acquisition after 64 rows. The experimental scaling factor was determined by comparing the PISEMA with a separated local field spectrum (Waugh, 1976) on a crystal of ^{15}N-acetyl leucine.

Additional magic angle spinning NMR spectra of gramicidin reconstituted in DMPC membranes or tyrocidin powders were recorded on wide-bore Bruker Avance solid-state NMR spectrometer operating at 9.34 or 17.5 Tesla.

Results and discussion

The growth rate of two different Gram-positive *Bacillus brevis* strains was tested in media of different composition. The optical densities of the cultures at 600 nm was taken as an indicator of cell density. The strain ATCC-8185 (also known as BG or DSM-362) produces high amounts of tyrothricin in a medium of gelatine or milk (not shown). As isotopic enrichment of these media is very difficult, if not impossible, other media are required to promote bacterial growth as well as the production of gramicidin. We, therefore, tested several common media, which are available in isotope enriched forms. These are based on peptones of different sources, yeast extracts, mixtures of amino acids or minimal media (NH_4Cl as the sole nitrogen source, or supplemented by single amino acids). As our initial attempts using strain DSM-362 to produce high amounts of gramicidin A were not satisfactory (Table 1) we included DSM-5618 in this study. This strain was first isolated from Turkish cheese and produces compounds which have the same crystalline structure, analytical composition, and biological properties as gramicidins and tyrocidines (Dubos and Hotchkiss, 1941). However, to our knowledge a more detailed analysis of the composition of these products is not available from the literature.

Table 1 shows OD_{600} values from the bacterial suspension after seven days of incubation at 37 °C when either strain DSM-362 or DSM-5618 is used. The production of tyrothricin was estimated by measuring the OD_{280} of the ethanol extract. A comparison of growth characteristics and tyrocidin production indicates that the strains DSM-362 and DSM-5618 have different phenotypes. Although the strain 5618 grows better on all media tested, this by itself cannot be taken as a measure for tyrothricin production. The concentration of tryptophan-containing peptides is more reliably monitored, however, by measuring the optical density at 280 nm. According to early reports *Bacillus brevis* does not produce tyrothricin in rich media (Stokes and Woodward, 1943). We were, therefore, surprised to observe that both strains produce the highest amounts of tyrothricin by in media like peptone from *E. coli* and yeast, peptone from *E. coli* or a mixture of twenty amino acids ('*All-AA*'). Notably, we observed good bacterial growth but almost no tyrothricin production when bacterial cultures in rich media were permanently agitated. This result suggests that sufficiently anaerobic conditions are required for gramicidin and tyrocidin production.

We also tested base media supplemented with single amino acids. A medium that contains asparagine as the sole nitrogen source readily supports gramicidin production (not shown), in agreement with earlier reports (Stokes and Woodward, 1943). In order to investigate if it was possible to biochemically label selected sites of the gramicidin backbone, certain media supplemented with DL-leucine, DL-valine, DL-isoleucine or glycine were also tested. These amino acids are much easier to label than asparagine, but unfortunately, they did not support growth of *B. brevis* (Figure 1). Similarly, only small amounts of tyrothricin are produced in media with NH_4Cl as the only nitrogen source (Figure 1).

Proline is present in tyrocidine but not in gramicidin. The proline analogue pipecolic acid was reported to decrease the ratio of tyrocidin/gramicidin by selectively blocking the synthesis of the former peptide (Okuda et al., 1963). Therefore, a medium was prepared from a mixture of the common amino acids, but proline was replaced by a four-fold amount of pipecolic acid. Similar tyrocidin to gramicidin ratios were obtained irrespective of whether pipecolic acid was present or not. Growth of *B. brevis* was virtually absent when pipecolic acid was offered as a sole nitrogen source (Figure 1).

Celtone is a commercially available rich medium, which is obtained from bacterial and yeast cultures. Whereas the strain DSM-5618 shows growth characteristics similar to that observed for the peptone/yeast medium, the strain DSM-362 does not grow in this environment (Figure 1). Martek, another commercially available medium, is obtained by partial hydrolysis of algal proteins. Although growth of both strains is good on this medium, tyrothricin production is virtually absent (Figure 1). Notably, whereas similar ratios of gramicidin/tyrocidin are produced in most media tested, only tyrocidin, but no gramicidin, was obtained using the Martek or the minimal NH_4Cl medium. Fur-

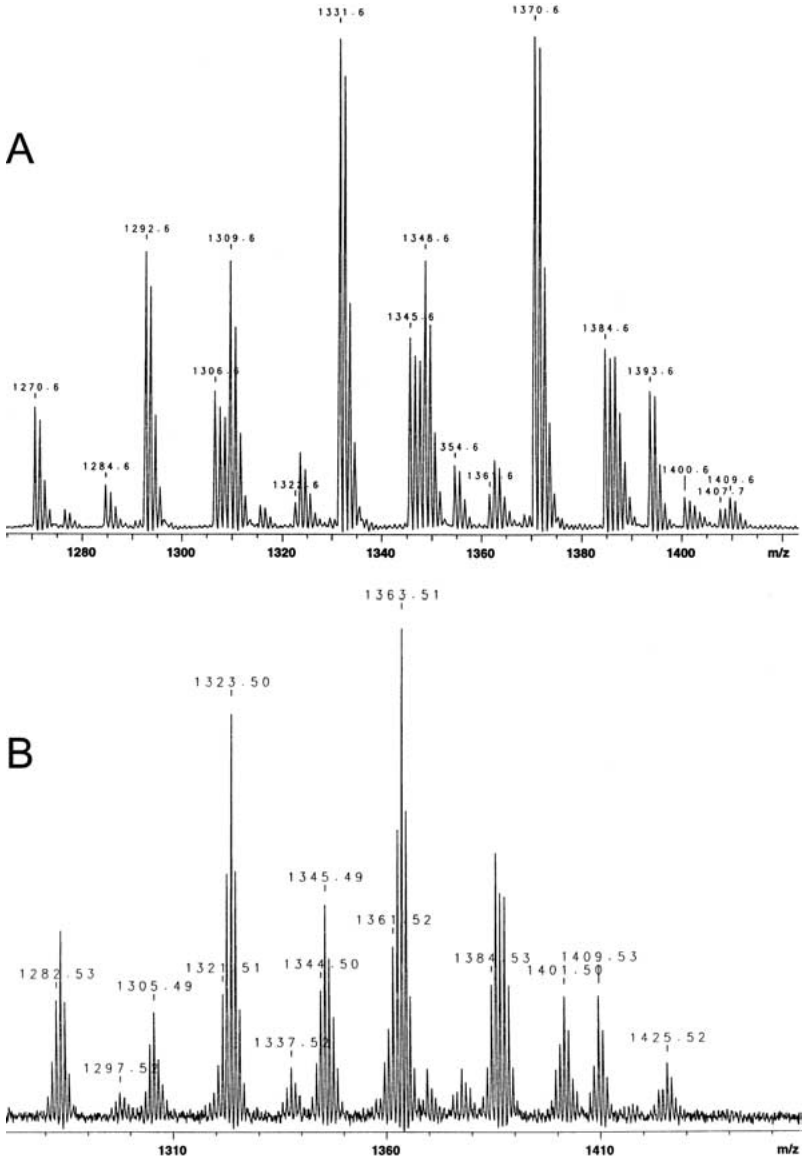

Figure 4. MALDI MS spectra of tyrothricin preparations. (A) Commercial product. (B and D) Ethanol extract of Culture-TC fermented in ^{15}N-Martek9 medium. (C) Ethanol extract from culture-TC grown in ^{15}N/^{13}C Celtone medium.

thermore, using different media produces differences in the distribution of tyrocidins but not of gramicidins.

Figure 2 shows HPLC separations of the ethanol extract (tyrothricin) obtained from cultures of *B. brevis* DSM-5618. Whereas the different gramicidin analogues separate well on a normal phase silica column (Figure 2A), the tyrocidin analogues are well resolved by C18 reversed phase HPLC (Figure 2B). The details of these purification steps are listed in the Materials and Methods section. The results from the HPLC analysis are also summarized in Table 1.

The products of the ethanol extract are identified by ^{1}H NMR in DMSO-d_6 and MALDI-MS. Figure 3A shows the ^{1}H spectrum of commercially available gramicidin A (Sigma) in DMSO-d_6. For assignment of all resonances the reader is referred to (Hawkes et al., 1987). Figures 3B and 3C show ^{1}H spectra of gramicidin A with and without ^{15}N decoupling, respectively. The peptide was obtained from the strain DLM-5618 grown on a medium prepared from *E. coli* peptone and yeast extract. The resonance of the hydroxyl proton of the C-terminal ethanolamine (marked by a star) has

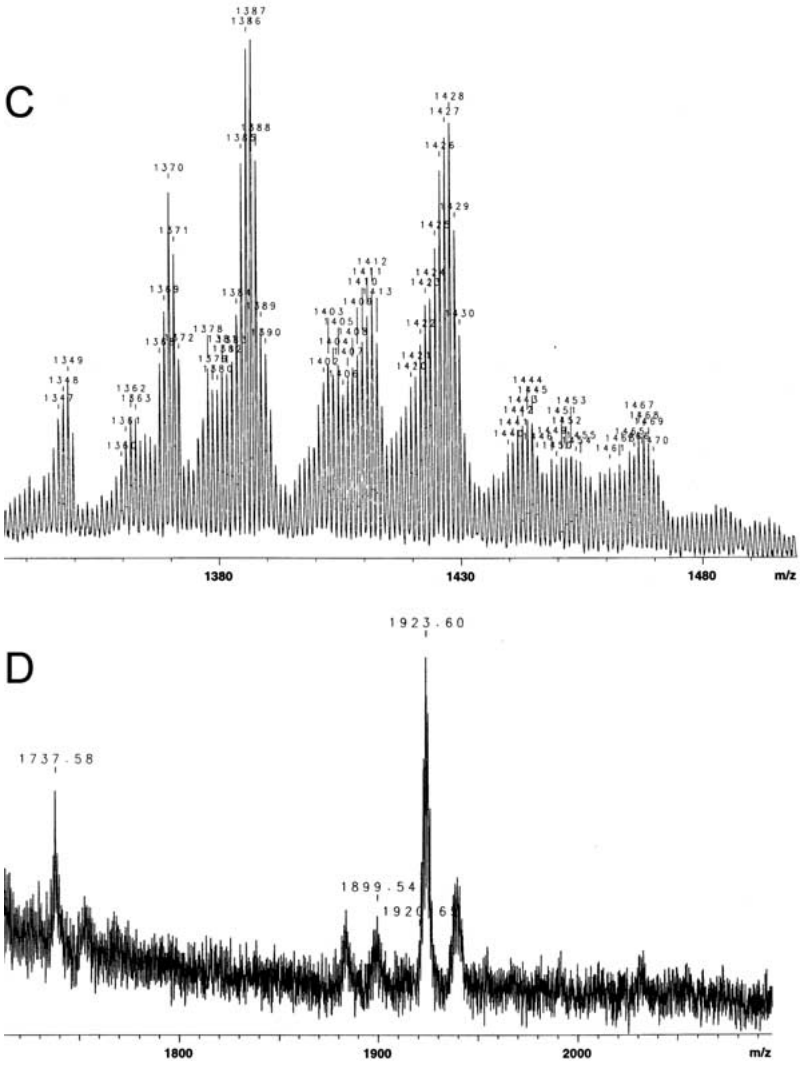

Figure 4. Continued.

shifted from 4.7 ppm to 2 ppm when the spectra of the commercial and of the biosynthetic product are compared (Figures 3A and 3B). We believe that this is due to a reduced amount of water in the preparation obtained from the strain DLM-5618, and further illustrated by the absence of the water peak at 3.3 ppm (cf. Figures 3A and 3B).

Mass spectrometric investigation of the commercial tyrocidin preparation (Sigma) reveals predominant peaks at 1271/1293 (tyrocidin A and its Na^+ complex), 1310 / 1332 (tyrocidin B $\pm$ Na^+) and 1349 / 1371 (tyrocidin C $\pm$ Na^+) at relative intensities of 0.64, 1.0 and 1.0 respectively (Figure 4A). The mass difference Δm of 39 between these tyrocidins is indicative of Phe $\rightarrow$ Trp substitutions. Furthermore, Tyr $\rightarrow$ Trp (Δm = 23) and Val $\rightarrow$ Ile (Δm = 14) derivatives of each of these compounds are detectable at approximately 15 and 40% intensity of the parent compound respectively.

The major m/z values of tyrocidin obtained from the strain DSM-5618 are 1309 (tyrocidin B), 1348 (tyrocidin C) and 1371 (tyrocidin C, Y$\rightarrow$W), with relative intensities of 0.53, 1 and 0.43 respectively (not shown). Analysis of these masses indicate a preference for a $Phe_6 \rightarrow$ Trp substitution, as observed in tyrocidin B, an additional $Phe_7 \rightarrow$ Trp substitution as observed in tyrocidin C, and a $Tyr_{10} \rightarrow$ Trp modification of tyrocidin C respectively. This latter modification is also observed in commercial tyrocidin but only as a small component. The $Val_1 \rightarrow$ Ile substitutions are only ob-

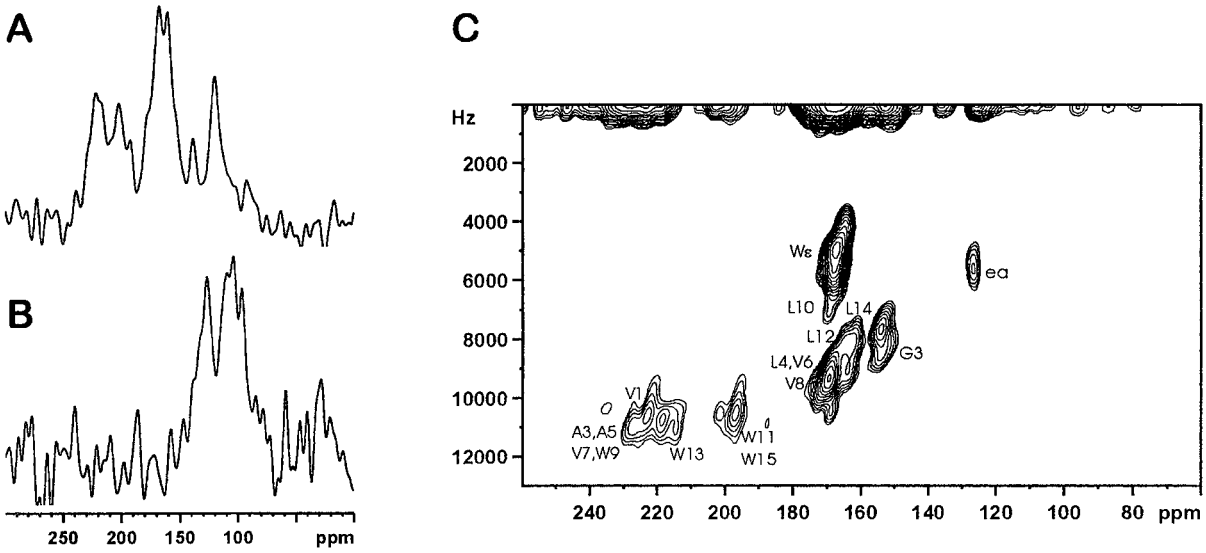

Figure 5. Proton-decoupled ^{15}N solid-state NMR spectra of (A) ^{15}N-Val1-gramicidin A. (B) ^{15}N-tyrocidin. Both peptides were reconstituted into oriented DMPC bilayers and equilibrated at 100% r.h. (37 °C). (C) 3π-2π PISEMA spectrum of gramicidin A correlating the ^{15}N chemical shift with the ^{1}H-^{15}N dipolar coupling. Also shown is the tentative assignment of resonances by comparing the chemical shifts and dipolar couplings to those obtained from single-site labelled residues (Ketchem et al., 1993; Tian et al., 1996; Tian and Cross, 1998), ea: ethanolamide. The spectra are calibrated with $^{15}NH_4Cl$ (41.5 ppm).

served in minor amounts. The sequence of the major tyrocidin analog from strain DSM-5618, therefore, is that of tyrocidin C.

-Val-Orn-Leu-Phe-Pro-Trp-Trp-Asn-Gln-Tyr-.

The m/z value of the ^{15}N-perlabelled tyrocidin obtained from strain DSM-5618 using the ^{15}N-Martek 9 medium as described above is 1363.5 (Figure 4B), which agrees with the expected increase by 15 units when compared to unlabelled tyrocidin C (10 amide, 2 indole and 3 amine nitrogens).

We also prepared a small amount of doubly labelled $^{13}C/^{15}N$ tyrothricin using the medium provided by Celtone. The observed increase in molecular weight when compared to unlabelled or ^{15}N-labelled peptides indicates a high degree of uniform isotopic labelling throughout the thyrocidin sequences (Figure 4C)

When the major gramicidin component of the unlabelled commercial product is analysed, an m/z value of 1904 is obtained by MALDI-MAS (not shown). The m/z value for the major gramicidin component obtained from extracting the growth product of strain DLM-5618 is 1903.7, i.e., identical within experimental error. Other m/z values observed in both samples are 1881 (gramicidin C) and 1919 (gramicidin A, Ile_1). The corresponding m/z value obtained from the expression in uniformly ^{15}N labelled medium is 1923 (Figure 4D). This is in excellent agreement with the expected increase of 20 mass units when all nitrogens of gramicidin A are labelled with ^{15}N (15 amino acids amides, the C-terminal ethanolamide and four indole nitrogens).

Figure 5 shows ^{15}N solid-state NMR spectra of biochemically prepared polypeptides reconstituted in oriented DMPC membranes at 310 K. Most of the signal intensity occurs in the region of transmembrane helical amide bonds. This is in agreement with the published gramicidin structure obtained from chemically synthesized polypeptides, the latter having previously been labelled with stable isotopes (^{2}H, ^{13}C, ^{15}N) at only a single or a few selected sites (Cross, 1997). Six major resonances are resolved from this uniformly ^{15}N labelled peptide sample when reconstituted in uniaxially oriented membranes, again agreeing with previous publications (Nicholson et al., 1987). It should be noted that the signal intensities are strongly dependent on the conditions of cross polarization and, therefore, only represent the number of underlying ^{15}N sites in a semi-quantitative manner. The spectrum obtained from uniformly ^{15}N labelled tyrocidin exhibits a much narrower chemical shift distribution around 120 ppm. This chemical shift frequency is indicative of either NH vectors being oriented close to

the magic angle or, alternatively, due to fast motional averaging. The spectrum of gramicidin A and tyrocidin C reflect the different conformational properties of these two polypeptide chains. Whereas gramicidin A is a linear pentadecapeptide, tyrocidin exhibits a circular arrangement of 10 amino acids. A detailed structural evaluation of the molecular structure of tyrocidin by solid-state NMR spectroscopy, however, requires further investigation and is not the subject of this paper. Although only 20 nitrogens are labelled, two-dimensional solid-state NMR experiments (PISEMA) cannot resolve all sites. Some improvement in resolution is however observed when compared to the one-dimensional spectrum (Figure 5C). This is a result of the lack of orientational dispersion within the gramicidin structure which is further enhanced by motional averaging (Davis and Auger, 1999; Fu and Cross, 1999).

In this paper we have described several strategies for obtaining gramicidin and tyrocidin polypeptides uniformly labelled with stable isotopes. The methods employed are very cost-efficient and should be feasible for producing the peptides in most laboratories. This contrasts with the solid-phase peptide synthesis of gramicidin A, which is very expensive especially when many sites are to be labelled with isotopes. In addition, the abundance of D-amino acids as well as the many hydrophobic residues makes the chemical synthesis more difficult than that of water-soluble peptides of a similar size (Fields et al., 1989). However, the preparation of isotope-enriched media is possible at relatively low cost using *E. coli* and yeast peptones as major constituents. Isotopically enriched *E. coli* biomass suitable for *B. brevis* fermentations can even be obtained as waste product during the expression of other proteins (e.g., Lambotte et al., 1998). The fact that is is possible to obtain large quantities of peptides, a high degree of labelling and the pure compounds using established HPLC methods make these peptides very useful for the application and development of one- and multi-dimensional solid-state NMR techniques.

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