7-O-malonyl Macrolactin A, a new macrolactin antibiotic from *Bacillus subtilis*, active against methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant enterococci, and a small colony variant of *Burkholderia cepacia*.

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Running Title: New Macrolactin active against multi-resistant bacteria

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Abstract

We report here the discovery, isolation, and chemical and preliminary biological characterization of a new antibiotic compound, 7-O-malonyl macrolactin A (MMA), produced by a Bacillus subtilis soil isolate. MMA is a bacteriostatic antibiotic that inhibits a number of multi-drug resistant Gram positive bacterial pathogens, including methicillin-resistant Staphylococcus aureus and vancomycin-resistant enterococci, and a small colony variant of Burkholderia cepacia. MMA-treated staphylococci and enterococci cells were pseudo-multicellular and exhibited multiple asymmetric initiation points of septum formation, indicating that MMA may inhibit a cell division function.
Introduction

The spread of resistance to antibiotics undermines the therapeutic utility of anti-infective drugs in current clinical use (1). For example, *Staphylococcus aureus*, a major cause of community and hospital-acquired infections, has developed resistance to most classes of antibiotics. Methicillin-resistant *S. aureus* (MRSA) strains appeared in the hospital environment after introduction of the semi-synthetic penicillin, methicillin, leaving vancomycin as the last line of defence for MRSA treatment (7). Vancomycin-intermediately-susceptible *S. aureus* were first isolated in 1997 in Japan (12) and later in other countries (8). With the recent appearance of vancomycin-resistant clinical isolates (32, 36, 38), no antibiotic class is effective against multi-resistant *S. aureus* infections. The increase of vancomycin-resistant enterococci (VRE), important agents of nosocomial infections, is another cause of great concern (2, 3, 19, 27). Therapy options for multi-resistant Gram-negative opportunistic bacterial pathogens are also diminishing. Such bacteria, like *Pseudomonas aeruginosa* and *Burkholderia cepacia* (6), are common environmental organisms and opportunistic pathogens having the capacity to infect essentially all tissues of patients with compromised host defences (21).

Compounding the problem of genetically-determined transmissible antibiotic resistance is the development of phenotypically-resistant, often slow-growing, forms in chronic bacterial infections. These may take the form of biofilm microbes or small colony variants (SCV; 13, reviewed in 15), are known for both Gram positive and Gram negative pathogens, and are usually associated with a worsening of the disease prognosis.

Thus, new antibiotics and therapy options are urgently needed to improve the management of bacterial infections (29, 35), and a major challenge is to find drugs that act against SCVs and/or bacteria growing in biofilms.
In this study, we report the discovery and preliminary characterization of 7-O-malonyl macrolactin A, MMA, a new antibiotic having bacteriostatic activity against clinical strains of MRSA, VRE and a SCV of *Burkholderia cepacia*.

**MATERIALS AND METHODS**

**Strains and media.** Microorganisms used to assess the antimicrobial activity of the macrolactins were from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany), numbered as DSM, and the American Type Culture Collections (ATCC, Rockville, MD, USA): methicillin-sensitive *S. aureus* (MSSA) strains (DSM 1104 and DSM 2569), *Escherichia coli* (DSM 498), *P. aeruginosa* (DSM 1117), *Enterococcus faecalis* (ATCC 29212), *Candida parapsilosis* (DSM 5784), *C. krusei* (DSM 6128), *C. albicans* (DSM 11225); and clinical isolates: MSSA strain 32, MRSA strain 2 and MRSA strain 3, *E. faecalis* strain E305 (vancomycin-resistant/ampicillin-sensitive; VRAS), *E. faecium* strain E315 (vancomycin-resistant/ampicillin-resistant; VRAR). The parental type (WT) and SCV pair of *P. aeruginosa* and *B. cepacia*, as well as the *Stenotrophomonas maltophilia* strain 1124, were isolated from cystic fibrosis patients in the Department of Medical Microbiology at the Medical School of Hannover (Germany). The *B. cepacia* WT strain 139 was isolated from a bronchoalveolar lavage and its SCV form 141 from a blood sample from the same patient. *P. aeruginosa* WT strain 134 and its SCV form 137 were isolated from the same respiratory tract specimen. The clonal identities of the SCVs and WTs were determined by pulsed-field gel electrophoresis, as previously described (13, 14).

The antibiotic producing bacterium, ICBB 1582, was isolated from a soil sample obtained from a farmyard at Takalar, South Sulawesi Province, Indonesia, in April 2000. The strain was deposited in the DSMZ under the accession number.
DSM 16696. Biochemical and phenotypic characterisations were made using Api 20E and 50CH kits (Biomerieux, Marcy l'Etoile, France). 16S rRNA sequence determination and analysis, performed as previously described (40), identified the organism as a *Bacillus subtilis* strain. All organisms were maintained in glycerol broth at -80°C.

Bacteria were cultivated overnight at 37°C on Luria-Bertani (LB) agar, except staphylococci and enterococci, which were grown on Columbia blood agar (BD, NJ, USA). Yeast strains were grown in DSM M186 medium (http://www.dsmz.de/microorganisms/html/media/medium000186.html). The medium used for growth of *B. subtilis* strain DSM 16696 and production of secondary metabolites had the following composition: yeast extract (Difco Laboratories, Detroit, Mich.) 5g/l, tryptone (Difco) 20g/l, NaCl (Merck, Darmstadt, Germany) 5g/l, glucose 5 (Merck), and Amberlite XAD-16 adsorbent resin (Rohm & Haas, Frankfurt, Germany) 40 g/l.

**Antibiotics.** Erythromycin (ERY), Vancomycin (VAN), Ampicillin (AMP), Gentamicin (GEN) and Miconazole (MCZ) were obtained from Sigma-Aldrich (Schnelldorf, Germany). Stock solutions, 10 mg/ml, were freshly prepared in sterile distilled water, except for erythromycin, which was prepared in ethanol. Erythromycin discs were obtained from A/S Rosco (Taastrup, Denmark) and contained 78 µg of diffusible antibiotic. Vancomycin and Ampicillin discs were obtained from BBL (NJ, USA) and contained respectively, 30 and 10 µg/disc.

**Cultivation of the Bacillus subtilis DSM 16696 strain and extraction of secondary metabolites.**

The preparation and recovery of secondary metabolites was carried out essentially as described by Sasse et al. (31). Briefly, this involved the inoculation of 500 ml Erlenmeyer flasks containing 200 ml of medium described above with
0.5 ml quantities of an overnight culture of *B. subtilis* DSM strain 16696 and incubation with shaking at 30°C for 7 days. The adsorbent resin was recovered from the culture broths by decantation, transferred into a column, and washed with 50% aqueous methanol (MeOH). Adsorbed products were subsequently eluted with 100% MeOH and, after evaporation, the remaining aqueous mixture was extracted four times with ethyl acetate. Evaporation of the solvent under reduced pressure yielded approximately 300 mg of oily residue from a total culture volume of 4l. This residue was re-suspended in 10 ml of MeOH and the solution extracted four times with the same volume of n-heptane to remove lipophilic products and contamination. The MeOH phase was retained for the isolation of natural products.

**Purification of macrolactins.** Macrolactins were purified by preparative RP-HPLC using a Nucleosil 100-7 C18 column (250/21 mm, Macherey-Nagel, Düren, Germany), a gradient of solvents: A (0.5% acetic acid/MeOH 51%) and B (0.5% acetic acid/MeOH 56%), gradient 0% B to 100% B in 60 min, a flow rate of 30 ml/min and UV detection at 280 nm. Between 40 and 60 mg of extract in 0.2 ml MeOH were used for the injection. Each macrolactin (5-7 mg) was further purified by LH-20 chromatography (column 760/25 mm, solvent MeOH/dichloromethane (1:1), flow rate 5 ml/min).

**Spectrometric analyses and structure determination.** HPLC-UV-MS analysis was performed on an HP 1100 HPLC system (Hewlett-Packard, Palo Alto, USA) with UV diode array detector and PE Sciex API 2000 LC/MS/MS system with an ACI device (Perkin-Elmer, Boston, USA), using a Nucleosil 120-5 RP C18 column (125/2 mm, Macherey-Nagel), at 40°C. MS data were obtained on a MAT 95 mass spectrometer in EI and DCI mode (Finnigan, USA). Active compounds/peaks were identified with their molecular masses and UV data by
searches in “Dictionary of Natural Products” database (Chapman and Hall/CRC), Antibase 2000 (VCH Wiley), and CrossFire Beilstein databases (MDL). For nuclear magnetic resonance (NMR) spectroscopy, the samples were dissolved in 99.95% MeOH-d₄, chloroform-d₃ or dichloromethane-d₂ and the data were obtained with an AVANCE DMS-600 spectrometer (Bruker, Karlsruhe, Germany). Optical rotation and UV spectrum were measured in UV MeOH (Merk, Darmstadt, Germany) on a Polarimeter MC 241 (Perkin Elmer) (d = 10 cm) and a UV-2102 PC UV-VIS Scanning Spectrophotometer (Shimadzu, Columbia, USA).

**Determination of anti-bacterial activities by the agar diffusion method.** Sterile discs (Schleicher & Schuell, Dassel, Germany) containing 10 µl of crude extract or 10 µl of purified macrolactin in MeOH (50 µg final compound concentration on the disc) were placed on fresh plates of Mueller-Hinton (MH) agar (Difco) seeded with suspensions (10⁵ CFU/ml) of overnight cultures of the test microorganisms. The diameters of the zones of inhibition of growth around the discs were measured after incubation periods of 18 h at 37°C.

**Minimal Inhibitory Concentrations.** The MICs were determined by the broth microdilution method as recommended by the National Committee for Clinical Laboratory Standards (24) using MH broth (MHB). Microtiter plates containing 50 µl of serial twofold dilutions of each antimicrobial agent per well, were inoculated with 50 µl of a bacterial suspension to yield a cell density of 5 x 10⁵ CFU/ml. MeOH and MHB alone (7-12 µl) had no effect and were used as controls. The microtiter plates were incubated for 48 h, visible growth and OD at 650 nm were recorded after 18, 24, 30 and 48 h of incubation and read with a microtiter reader 3550-UV (Biorad, Munich, Germany). The MIC was the lowest antibiotic concentration that completely prevented visible growth after
incubation at 37°C for 18 h; the Minimum Restrictive Concentration (MRC) was defined as the lowest antibiotic concentration that caused at least 50% retraction of growth at 37°C for 18 h (by visual observation and OD at 650 nm).

**Kinetics of growth at sub-inhibitory concentrations.** Bacterial growth at sub-MICs of 7-O-malonyl-macrolactin A was also investigated. Overnight cultures were used to inoculate fresh MHB containing MMA at different sub-MICs to a cell density of \(\sim 10^6\) CFU/ml and the cultures were incubated at 37°C with gentle shaking. Aliquots were removed at 0, 1, 2, 4, 6, 8 and 24 h and dilutions were plated in LB agar medium using a spiral plater (Spiral Biotech, Norwood, USA). The plates were incubated for 24 h at 37°C and the number of colonies developing was counted with a laser colony counter (CASBA 4, Spiral Biotech).

**Sub-MIC effects.** Sub-MICs effects (SME) were determined by the postantibiotic effect (PAE) method of Craig and Gudmundsson (5). Briefly, 7-O-malonyl macrolactin was added at sub-MICs to tubes containing MHB, to which either MRSA 3 or VRAR *E. faecium* E315 at \(\sim 10^6\) CFU/ml was subsequently added. Medium without antibiotic was used as a control. After incubation for 1h at 37°C, samples were diluted 1:1000 in warmed drug-free MHB to dilute out the antibiotic and incubated further at 37°C. Viability counts were made before exposure, immediately after dilution (0 h), and then hourly, by plating on LB agar plates as described above. The SME was defined by the relationship \(\text{SME} = \text{Ts} - \text{C}\), where \(\text{Ts}\) is the time it takes for cultures exposed to sub-MICs to increase 1 \(\log_{10}\) unit above the counts observed immediately after antibiotic removal by dilution, and \(\text{C}\) is the corresponding time for the unexposed control.
**Cytotoxicity assays.** HeLa human epithelial cells and L929 mouse fibroblasts, obtained originally from the ATCC, were cultured in DMEM (Gibco BRL, Life Technologies, Karlsruhe, Germany) low (1 mg/l) and high (4.5 mg/l) glucose, respectively, supplemented with 10% (v/v) foetal bovine serum (Gibco), at 37 °C in a 5% CO₂ atmosphere. Cell suspensions were obtained by treatment of monolayer stock cultures with 0.25% trypsin, with and without 0.2 g/l of EDTA (Gibco), for HeLa and L929, respectively, diluted to obtain suspensions of 2 × 10⁵ cells/ml and 100 µl added to Nunc 96 well microtiter plates containing or not serial dilutions of macrolactins in MeOH (100 µl). Dilutions of MeOH and cell culture medium were used as controls: no effect of these solutions on cell growth was observed. Morphological changes in cells after exposure to the compounds for 1, 2 and 5 days were assessed by phase-contrast microscopy. Cell counts after 5 days of exposure were also made using the CyQUANT cell proliferation assay (Molecular Probes, Eugene, USA), a highly sensitive, fluorescence-based microplate assay for determining numbers of cultured cells (17), that employs CyQUANT dye, which produces a large fluorescence enhancement upon binding to cellular nucleic acids that can be measured using fluorescein excitation. The fluorescence emission of the dye-nucleic acid complexes correlated linearly with the cell number. The linear range of the assay under our experimental conditions was 50 to 250,000 cells per 200 µl sample. For this test, the supernatant fluid was carefully removed, the cells were washed with PBS, the buffer was removed and cells frozen at -80°C. For the assay, cells were thawed at room temperature and lysed in buffer containing the CyQUANT dye prepared according the manufacturer's instructions. Fluorescence was measured with a fluorometric plate reader (Titertex Fluoroskan II; excitation, 480 nm; emission, 520 nm). The values obtained were used to calculate the percentage of inhibition of cell proliferation in the
presence of macrolactins, according to the formula: 100 - [(cell growth in the presence of drug / cell growth in drug free medium) x 100].

**Transmission electronic microscopy.** For transmission electron microscopy (TEM), the macrolactin compounds were added at sub-MICs to tubes containing MHB, inoculated with either MRSA 3 or VRAR *E. faecium* E315 at densities of ~10^6 CFU/ml, and incubated at 37°C for 4 h. Tubes without antibiotic were used as controls. The cells were harvested by centrifugation at 12000 rpm and fixed with 1.0% (v/v) glutardialdehyde in phosphate-buffered saline (PBS), pH 7.0, and processed as previously described (40). The samples were examined using a energy filter transmission electron microscope (Zeiss CEM 902, conventional mode, 30 µm objective aperture, 80 kV acceleration voltage, Zeiss Oberkochen, Germany). Electron micrographs were recorded digitally with a high resolution 1024 x 1024 CCD camera (Proscan, Electronic Systems, Scheuring, Germany).

**RESULTS**

**Producer strain DSM 16696.** The DSM strain 16696 was characterised as a gram-positive rod (0.5-1µm x 1-3 µm), motile with flagella, forming endospores (d. ±0.2 µm), and producing opaque milky white with undulating and roughly edged colonies. In the API system, positive reactions were oxidase, ornithine, mannitol, Voges Proskauer, citrate, tryptophan deaminase and hydrolysis of starch, whereas negative reactions were nitrate, lysine, H₂S production, glucose, xylose, beta galactosidase, indole and urease. According to the biochemistry tests and 16S rRNA sequence homology searches in the FASTA system, DSM 16696 is a strain of *B. subtilis* (99% 16S rRNA sequence identity to *B. subtilis* strain accession no AY775778; Gene Bank/EMBL/DDBJ).
Isolation of three macrolactin compounds. Secondary metabolites produced *B. subtilis* strain DSM 16696 were obtained from small scale cultures and screened for antimicrobial activities, as described in Materials and Methods. HPLC fractionation of the XAD extracts, and identification of the biologically-active fractions by the bioassays, revealed ten different antibacterial compounds, which were identified by their UV and MS spectra as bacillomycins, oxydifficidins, aromatic lipopeptides, and a new compound, together with two other known compounds belonging to the macrolactin group. Since the initial screening revealed the new compound to be inhibitory towards MRSA and VRE, subsequent efforts focused on its characterization. And, since it was subsequently identified as a macrolactin, the three macrolactins (compounds I, II and III) were isolated from a 4l culture, purified by preparative RP-HPLC and LH-20 chromatography and compared.

**NMR-data of compound II, the new macrolactin:** NMR analysis was performed on the purified macrolactin obtained by RP-HPLC and LH-20 chromatography. The data obtained were: $^1$H-NMR (600 MHz, methanol-d$_4$): δ [ppm], $^J$ [Hz] = 5.59 (d, $^J$=11.7, 2-H), 6.67 (t, $^J$=11.3, 3-H), 7.25 (dd, $^J$=14.7, 11.7, 4-H), 6.15 (dt, $^J$=15.4, 7.2, 5-H), 2.60 (m, 6-H$_2$), 5.50 (ddd, $^J$=6.0, 6.0, 6.0, 7-H), 5.75 (dd, $^J$=15.3, 5.5, 8-H), 6.71 (dd, $^J$=15.1, 11.3, 9-H), 6.13 (t, $^J$=10.2, 10-H), 5.63 (dt, $^J$=10.6, 8.4, 11-H), 2.63 (m, 12-Ha), 2.33 (ddd, $^J$=13.0, 7.2, 5.5, 12-Hb), 3.84 (ddd, $^J$=10.6, 6.0, 5.7, 13-H), 1.66 (m, 14-H$_2$), 4.39 (dt, $^J$=6.3, 6.3, 15-H), 5.60 (dd, $^J$=15.1, 6.4, 16-H), 6.21 (dd, $^J$=15.1, 10.6, 17-H), 6.10 (dd, $^J$=15.1, 10.6, 18-H), 5.69 (ddd, $^J$=14.9, 7.0, 6.8, 19-H), 2.23 (td, $^J$=14.0, 6.8, 20-Ha), 2.15 (td, $^J$=14.4, 7.2, 20-Hb), 1.54 (m, 21-H$_2$), 1.70 (m, 22-Ha), 1.62 (m, 22-Hb), 5.05 (ddq, $^J$=4.5, 7.1, 6.1, 23-H), 1.30 (d, $^J$=6.0, 24-H$_3$), 2.90 (m, 2'-H$_2$). $^{13}$C-NMR (150 MHz, methanol-d$_4$): δ [ppm] = 167.94 (C-1), 118.52 (C-2), 144.50 (C-3), 130.79 (C-4),
Identification of the new compound as 7-\textit{O}-malonyl macro lactin A (MMA). The chemical structures and physical properties of the three macro lactin compounds are compared in Fig. 1 and Table 1, respectively. The molecular ion \( m/z \) 402 and the UV absorptions at 227 and 261 nm enabled the identification of I as macro lactin A or its 10\( \varepsilon \)-isomer. The latter was ruled out by its optical rotation of \( [\alpha]_{22}^{20} = -138 \) compared to about \(-10\) found for macro lactin A. A direct comparison of the \(^1\)H and \(^{13}\)C NMR data of the macro lactin A (data not shown) produced by the DSM 16696 strain with the previous reported was hampered, because only \(^1\)H NMR data in benzene-\( d_6 \) and \(^{13}\)C NMR data in pyridine-\( d_5 \) are available in the literature (10).

Compound II was identified as a macro lactin A type from its identical UV spectrum. Mass spectrometry indicated the molecular mass of 488, which is 86 a.m.u. higher than the mass observed for I. Corresponding to the elimination of one \( \text{H}_2\text{O} \) from I, compound II showed the loss of malonic acid by a fragment-ion at \( m/z \) 383 in the (-)-ESI spectrum. The NMR data of II in MeOH-\( d_6 \) were nearly identical to I. However, compared to I the 7-\( \text{H} \) signal was shifted about 1.2 ppm down-field as consequence of the acylation of 7-\( \text{O} \). Because only one carboxy group was directly visible in the NMR spectra of II in MeOH-\( d_4 \), the residue at 7-\( \text{O} \) was identified by comparison with I from the \(^1\)H and \(^{13}\)C NMR spectra in dichloromethane-\( d_2 \). Here the malonyl residue was clearly indicated by additional carboxy \(^{13}\)C signals at 166.33 and 169.15 ppm and a methylene \(^{13}\)C signal at 42.17 ppm, which was correlated to two \(^1\)H doublet signals at 3.51 and
3.40 ppm ($J = 15.5$ Hz). The $^1$H NMR data of the compound III in CDCl$_3$ (data not shown) were found to be identical to those described by Jaruchoktaweechai et al. (16) for 7- O-succinyl macrolactin A (SMA).

7- O-malonyl macrolactin A (MMA) is a new bacteriostatic antibiotic active against MRSA, VRE and a small colony variant of B. cepacia. The agar diffusion method was used to compare the anti-microbial activities of the crude extract from strain DSM 16696, the three purified macrolactins and, as controls, relevant antibiotics in clinical use, against reference strains and clinical isolates (Table 2). All macrolactins showed good inhibition activity against both methicillin sensitive and MRSA but, whereas MMA and SMA also inhibited VRE, unsubstituted macrolactin A did not. However, the inhibition zones observed with the staphylococcal test strains were straight turbid, rather than clear, suggesting a growth inhibition rather than a bactericidal activity. To rule out the possibility of the turbidity results from the development of resistant variants, the small colonies developing in the inhibition zones were purified and re-tested for sensitivity. In all cases, they gave turbid inhibition zones, confirming that the macrolactins inhibit growth rather than kill (data not shown).

These qualitative experiments were subsequently confirmed by quantitative determination of MIC values, the antibiotic concentrations that totally prevent microbial growth in liquid cultures (Table 3). In fact, the MICs of MMA for staphylococci and enterococci were higher than 128 µg/ml, but, a strong inhibition of growth of both types of organism was observed at much lower concentrations. We therefore determined the lowest concentrations of the new compound that resulted in 50% inhibition of bacterial growth, the MRC (minimal restrictive concentration). The MRCs were between 1 and 64 µg/ml for the $S. aureus$ reference strains and for the MRSA strains; and between 0.06 and 4 µg/ml for $E. faecalis$ ATCC strain 29212 and the clinical isolates VRAS
E305 and VRAR E315 (Table 3). No changes in the end points occurred when the incubation was extended to 48 h, which shows that the bacteria inhibited by the drugs do not re-initiate growth (data not shown). The liquid growth test also confirmed that MMA and SMA exhibited higher activities than MA, against MRSA and VRE, suggesting the importance of substituents on the C-7 for the biological activity of this group of compounds (data not shown).

MMA was not active against the majority of the gram-negative isolates tested (data not shown). Interestingly, however, a small colony variant of *B. cepacia*, but not the parental normal colony type, was inhibited, as was a *Candida krusei* strain, but not other *Candida* strains tested (Table 3).

**Long term inhibition of staphylococci and enterococci at MMA sub-MICs.** To investigate the growth inhibition effect of 7-*O*-malonyl macrolactin A, time courses of the effects on bacterial viability of antibiotic at several sub-MIC values were followed (Fig. 2). MMA at 1 µg/ml, rapidly reduced the counted number of viable cells of the methicillin-susceptible *S. aureus* strain by almost 2 orders of magnitude over the first two hours of exposure. Over the next 6 hours, bacterial multiplication occurred at the same rate as that of the non-treated control; though levels remained about one log lower than those of the controls over the 24 hours period of the experiment. In the case of the MRSA 3 strain, no significant reduction in counts of viable cells occurred, but no significant growth was observed either, and by the end of the experiment there was a difference of >2 logs in the number of viable cells of the treated and control cultures. In the case of the *E. faecalis* ATCC strain 12912, the reduction in number of viable cells observed, ca. 50%, was less than that seen with the *S. aureus* reference strain, but otherwise the picture was similar. Also, MMA had an inhibitory effect on the VRAR *E. faecium* strain E 315 similar to that on the MRSA strain, namely complete inhibition of growth. Essentially, the same
patterns of viable cell numbers were seen with both sub-inhibitory concentrations of antibiotic, 4 and 16 µg/ml. Thus, interestingly, MMA was bacteriostatic for the antibiotic-resistant strains tested in liquid cultures but less inhibitory for the sensitive ones, though the sample is too small to generalize.

**MMA induces significant cellular damage at sub-MICs.** The PAE and sub-MICs effect (SME), the time taken to repair and recover from antibiotic damage, are important properties of antibiotics which reflect the degree of such damage inflicted by antibiotics, at supra-MICs and sub-MICs, respectively (5, 25). The SMEs for the VRAR *E. faecium strain E315* and MRSA strain 3 after 1 h of exposure to 16 µg/m MMA were 2.31 h and 0.42 h, respectively, indicating that the compound induced significant damage, particularly in enterococci, at sub-MIC concentrations.

**MMA inhibits separation of daughter cells.** MMA-treated cells of *E. faecium* VRAR E315 and MRSA 3 were examined by electron microscopy in order to obtain ultrastructural information about the damage caused. Sub-MICs of MMA had very marked effects on cell division and separation of daughter cells was severely inhibited through incomplete septum-formation (Fig. 3 and 4). Treated cells of MRSA 3 were larger than controls, and approximately 60% were observed in packets of non-separated cells (data not shown), in which multiple asymmetric initiation points of septum formation were visible (Fig. 3 d, e and f). Similarly, treated *E. faecium* VRAR E315 showed chains of non-separated cells, in which several symmetric initiations of cell division are evident (Fig. 4 d, e and f). Moreover, treated cells of *E. faecium* had a smooth appearance whereas untreated cells had rough surfaces (Fig. 4 c and e). SMA,
but not MA, induced similar morphological alterations in both MRSA and VRE, but to a lesser extent (data not shown).

**MMA exhibits weak cytotoxicity.** Macrolactin A has previously been reported to exhibit cytotoxic and antiviral activities (10) and, as described above, MMA exhibited weak activity towards a *Candida krusei* strain. Possible activity of MMA towards mammalian cells was therefore assessed with L929 mouse fibroblasts and HeLa human epithelial cells. As can be seen in Fig. 5, all three macrolactins inhibit the growth of L929 mouse fibroblasts, with MA being the most active, which suggests that eukaryotic cytotoxicity may be modulated by varying the C-7 substituent. 7-\(\text{O}\)-malonyl macrolactin A was less inhibitory for the human than the mouse cells tested: quantitation of the cytotoxic effect by means of the CyQuant assay revealed that MMA partially inhibited the proliferation of HeLa cells at 31.25 \(\mu\)g/ml and totally at 62.5 \(\mu\)g/ml (Fig. 5). The anti-proliferative effect of the macrolactins was reflected in a change in cell morphology to rounded cells.

**DISCUSSION**

Macrolactins are macrolides containing three separate diene structure elements in a 24-membered lactone ring (10), previously reported to be produced by a *Bacillus* sp., an unclassifiable marine bacterium, and an *Actinomadura* sp. (10, 16, 18, 23). Six macrolactins were first described in 1989 by Gustafson et al. (10), and reported to have antiviral properties, with macrolactin A being the most active compound of the group. In 2000, Jaruchoktaweechai et al. (16) subsequently isolated three further macrolactins with weak antibacterial activities, and in 2001, Nagao et al. (23) reported the isolation of seven new macrolactins. Until now, sixteen macrolactins have been chemically described and macrolactins A and E have been chemically synthesized (22, 33, 34).
Macrolactins are considered to be potent antiviral and cytotoxic agents, which have also antibacterial activity (10, 16). Macrolactin A inhibits murine melanoma cancer cells in in vitro assays, replication of Herpes simplex viruses, and the enzyme squalene synthase (4), and protects T-lymphoblast cells against human HIV viral replication (10). Macrolactins also inhibit S. aureus and B. subtilis. Nagao et al. (23) compared the activities of 9 macrolactins and suggested that the hydroxyl group at C-15 may play an important role in the antibacterial activity of these compounds. To our knowledge, there is no information about the mechanism of action of this group of compounds.

The new macrolactin reported here, 7-O-malonyl macrolactin (MMA), and the 7-O-succinyl macrolactin (SMA) produced by the same strain, exhibited inhibition activity at low µg/ml concentrations against multi-resistant clinical isolates of S. aureus and enterococci. MMA also inhibited a small colony variant (but not the normal morphotype) of B. cepacia, the only Gram negative bacterium tested that was inhibited by MMA. The fact that SCV of P. aeruginosa were not inhibited suggests that MMA may not be generally active against slow growing bacteria, but rather have a specific activity against SCV of B. cepacia. This observation warrants further investigation.

The antibacterial activity of the new compound is bacteriostatic rather than bacteriocidal. In this context, however, it should be pointed out that other bacteriostatic agents, such as chloramphenicol, clindamycin, macrolides and linezolid, have been effectively used for treatment of a range of bacterial infections, including endocarditis, meningitis, and osteomyelitis (26, 28, 37). The possible influence of MMA on the production of virulence factors by MRSA and VRE remains to be investigated, as do potential synergies with other drugs.

Another property of MMA of clinical relevance is its sub-MICs effect (SME), which reflects the degree of antibiotic-inflicted bacteria, because viable but damaged bacteria are more susceptible to clearance (25). The SMEs for
the vancomycin- and ampicillin-resistant *E. faecium strain* E 315 and methicillin-resistant *S. aureus* strain 3 after 1 h of exposure to 16 µg/m MMA were 2.31 h and 0.42 h, respectively, indicating that the compound induced significant cellular damage, especially in enterococci, at sub-MICs alone.

Thought macrolactins have been known since the late 1980s, there are no reports on their mode of action. Electron microscopic analysis of MMA-treated MRSA and VRE strains ultrastructure revealed alterations in the septation process, indicating a direct or indirect inhibition of one or more stages in cell division. Morphological changes of this type are known to be induce by compounds that inhibit the cell wall synthesis, such as penicillins and compound P, the active principle of crude tea extract. MRSA and *S. aureus* grown in the presence of such compounds produce a thickened cell wall (9, 11). Abnormal morphology and the appearance of amorphous material on the surface of glycopeptide-treated bacteria were also described for *S. aureus* and enterococci (20, 30, 39). The mechanism by which 7-*O*-malonyl macrolactin A induces morphological changes is interesting because pseudo-multicellular clusters are unable to produce daughter cells and, as a consequence, should have a reduced ability to disseminate, and therefore be less virulent. The morphological alterations induced by MMA were not a unique property of this new compound, since they were also observed for the SMA. Since 7-*O*-malonyl-and-succynil-macrolactins analysed in this study showed superior antibacterial activity and lower cytotoxicity in comparison with macrolactin A, this indicates an important role of the C-7 residue in the biological activities of these compounds.

In conclusion, in this study we have described a new variant of macrolactin A, 7-*O*-malonyl macrolactin A, and showed it to inhibit the growth of MRSA, VRE and a *SCV* of *B. cepacia*. Together with 7-*O*-succynil macrolactin A, it would see to hold promise for the development of new drugs against these clinically
problematic microbial pathogens. We have also provided evidence for a tentative target in cell wall biosynthesis, and this may ultimately lead to the identification of a new target for antimicrobial drug discovery programmes.

ACKNOWLEDGMENTS

We thank Victor Wray for helpful discussions and critical reading of the manuscript, Peter Golyshin and Rene Huppmann of the GBF for their help in HPLC analysis, and Elke Barth for technical assistance in electron microscopy. K. N. Timmis thanks the Fonds der Chemischen Industrie for generous support.

REFERENCES


Figure Legends

Figure 1. Chemical structures of the macrolactin compounds produced by *B. subtilis* DSM 16696.

Figure 2. Growth curves for bacteria treated with 7-*O*-malonyl macrolactin A (MMA) at sub-MIC levels. The curves are viable cell concentrations plotted against time. A: MSSA 32; B: MRSA 3; C: *E. faecalis* ATCC29212; D: *E. faecium* VRAR E315. The sub-MICs (MRC and 4x MRC) of MMA in µg/ml are indicated for each curve.

Figure 3. Transmission electron micrographs of MMA-treated methicillin-resistant *Staphylococcus aureus*. The MRSA strain 3 clinical isolate was treated for 4 h with 16 µg/ml of 7-*O*-malonyl macrolactin A (MMA). a, b and c are untreated controls and d, e and f are treated bacteria. Different states and positions of septum formation are indicated with large arrowheads (b, c, e and f). Asymmetrical initiations of septum formation are also observed in treated cells (small black arrowheads) in e and f.

Figure 4. Transmission electron micrographs of MMA-treated vancomycin- and ampicillin-resistant enterococcus. The *E. faecium* E315 clinical isolate was treated for 4 h with 16 µg/ml of 7-*O*-malonyl macrolactin A (MMA). a, b and c are untreated controls and d, e and f are MMA-treated bacteria. Septa are indicated with large black arrowheads (c, e and f). Asymmetric initiation of cell division (arrow) and pseudomulticellular chains (white asterisk) with many primordial septa are indicated (small black arrowheads) in treated cells (d-f).
Figure 5. Cytotoxicity of macrolactins. Effect of macrolactin A, 7-\(\text{O}\)-malonyl macrolactin A and 7-\(\text{O}\)-succinyl macrolactin A on L929 mouse fibroblasts and HeLa human epithelial cells.
Table 1. Physical properties of the three macrolactin compounds produced by *Bacillus subtilis* DSM 16696.

<table>
<thead>
<tr>
<th>Property</th>
<th>macrolactin A (I)</th>
<th>7-O-malonyl macrolactin A (II)</th>
<th>7-O-succinyl macrolactin A (III)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular formula</td>
<td>C_{24}H_{35}O_{5}</td>
<td>C_{27}H_{36}O_{8}</td>
<td>C_{28}H_{38}O_{8}</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>402.53</td>
<td>488.57</td>
<td>502.60</td>
</tr>
<tr>
<td>UV (MeOH) [\lambda_{\text{max}} \text{(lg } \varepsilon)]</td>
<td>227 (4.537)</td>
<td>227 (4.397), 230 (sh)</td>
<td>227 (4.596)</td>
</tr>
<tr>
<td>[\alpha^2_D \text{ (c in MeOH)}]</td>
<td>− 10.7 (0.68)</td>
<td>− 6.2 (0.63)</td>
<td>− 19.9 (0.7)</td>
</tr>
<tr>
<td>(−)-MS-ESI-TOF [m/z \text{ (%)}]</td>
<td>401.2 (38) [M−H]^−</td>
<td>487.2 (100) [M−H]^−</td>
<td>501.3 (100) [M−H]^−</td>
</tr>
<tr>
<td></td>
<td>437.2 (100) [M+Cl]^−</td>
<td>443.2 (44) [M−H−CO_2]^−</td>
<td>117.0 (12) [succinic acid−H]^−</td>
</tr>
<tr>
<td></td>
<td>803.4 (63) [2M−H]^−</td>
<td>383.2 (27) [M−H−malonic acid]^−</td>
<td></td>
</tr>
</tbody>
</table>
Table 2. In vitro antibacterial activities of the macrolactins compounds isolated in this study in comparison with the crude extract produced by DSM 16696 strain and reference compounds.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Inhibition zone size (mm)</th>
<th>Crude extract&lt;sup&gt;a&lt;/sup&gt;</th>
<th>I - macrolactin A&lt;sup&gt;b&lt;/sup&gt;</th>
<th>II - 7-O-malonyl macrolactin A&lt;sup&gt;b&lt;/sup&gt;</th>
<th>III - 7-O-succinyl macrolactin A&lt;sup&gt;b&lt;/sup&gt;</th>
<th>ERY&lt;sup&gt;c&lt;/sup&gt;</th>
<th>VAN&lt;sup&gt;d&lt;/sup&gt;</th>
<th>AMP&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSSA DSM 1104</td>
<td></td>
<td>28</td>
<td>18</td>
<td>26</td>
<td>28</td>
<td>30</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>MSSA 32</td>
<td></td>
<td>32</td>
<td>25</td>
<td>30</td>
<td>22</td>
<td>40</td>
<td>27</td>
<td>41</td>
</tr>
<tr>
<td>MRSA 2</td>
<td></td>
<td>24</td>
<td>27</td>
<td>40</td>
<td>37</td>
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<td>24</td>
<td>0</td>
</tr>
<tr>
<td>MRSA 3</td>
<td></td>
<td>28</td>
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<td>41</td>
<td>38</td>
<td>0</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td><em>E. faecalis</em> ATCC 29212</td>
<td></td>
<td>18</td>
<td>0</td>
<td>25</td>
<td>12</td>
<td>25</td>
<td>24</td>
<td>32</td>
</tr>
<tr>
<td><em>E. faecalis</em> VRAS E305</td>
<td></td>
<td>15</td>
<td>0</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>35</td>
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<tr>
<td><em>E. faecium</em> VRAR E315</td>
<td></td>
<td>40</td>
<td>0</td>
<td>15</td>
<td>20</td>
<td>25</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>B. cepacia</em> SCV 141</td>
<td></td>
<td>40</td>
<td>0</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>ND</td>
<td>0</td>
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</tbody>
</table>

<sup>a</sup>10 µl/disc, <sup>b</sup>50 µg/disc, <sup>c</sup>78 µg/disc, <sup>d</sup>30 µg/disc and <sup>e</sup>10 µg/disc.

MSSA: Methicillin-Sensitive *Staphylococcus aureus*

MRSA: Methicillin-Resistant *Staphylococcus aureus*

VRAS: Vancomycin-Resistant Ampicillin-Sensitive

VRAR: Vancomycin-Resistant Ampicillin-Resistant

SCV: Small Colony Variant

ND: not determined
Table 3. Antimicrobial activities of 7-O-malonyl macrolactin A and reference compounds against clinical and reference isolates.

<table>
<thead>
<tr>
<th>Strain</th>
<th>7-O-malonyl macrolactin A</th>
<th>µg/ml</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
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<tbody>
<tr>
<td>MSSA DSM 1104</td>
<td>&gt;128</td>
<td>64</td>
<td>1</td>
<td>0.5</td>
<td>0.125</td>
<td>0.25</td>
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<tr>
<td>MSSA DSM 2569</td>
<td>&gt;128</td>
<td>64</td>
<td>0.5</td>
<td>1</td>
<td>0.5</td>
<td>0.25</td>
<td>ND</td>
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</tr>
<tr>
<td>MSSA 32</td>
<td>&gt;128</td>
<td>1</td>
<td>0.125</td>
<td>0.06</td>
<td>0.06</td>
<td>0.25</td>
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</tr>
<tr>
<td>MRSA 2</td>
<td>&gt;128</td>
<td>4</td>
<td>2</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>128</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MRSA 3</td>
<td>&gt;128</td>
<td>4</td>
<td>1</td>
<td>64</td>
<td>&gt;128</td>
<td>128</td>
<td>ND</td>
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<tr>
<td><em>E. faecalis</em> ATCC 29212</td>
<td>128</td>
<td>4</td>
<td>2</td>
<td>0.5</td>
<td>2</td>
<td>32</td>
<td>ND</td>
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<tr>
<td><em>E. faecalis</em> VRAS E305</td>
<td>&gt;128</td>
<td>0.06</td>
<td>&gt;128</td>
<td>0.5</td>
<td>128</td>
<td>64</td>
<td>ND</td>
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<tr>
<td><em>E. faecium</em> VRAR E315</td>
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<tr>
<td><em>B. cepacia</em> WT 139</td>
<td>&gt;128</td>
<td>-</td>
<td>ND</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
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<td><em>B. cepacia</em> SCV 141</td>
<td>128</td>
<td>32</td>
<td>ND</td>
<td>128</td>
<td>128</td>
<td>&gt;128</td>
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<tr>
<td><em>C. parapsilosis</em> DSM 5784</td>
<td>128</td>
<td>-</td>
<td>ND</td>
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<td></td>
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<tr>
<td><em>C. krusei</em> DSM 6128</td>
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<td>32</td>
<td>ND</td>
<td>ND</td>
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<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. albicans</em> DSM 11225</td>
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<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>2</td>
<td></td>
<td></td>
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</table>

MRC (Minimal Restrictive Concentration): The Minimum Restrictive Concentration (MRC) was defined as the lowest antibiotic concentration that prevented more than 50% the growth of treated bacteria in comparison with the drug free bacterial growth at 37°C for 18 h (by visual observation and OD at 650 nm).

MIC and MRC endpoints for the *Candida* strains were read after 48 h.

ND, not determined
Fig. 1

Macrolactin A (I) $R = H$

7-Ο- malonyl macrolactin A (II) $R = \text{CO-CH}_2\text{-COOH}$

7-Ο- succinyl macrolactin A (III) $R = \text{CO-CH}_2\text{-CH}_2\text{-COOH}$
Fig. 2

A

S. aureus

Log_{10} CFU/ml

B

MRSA 3

Log_{10} CFU/ml

C

E. faecalis

Log_{10} CFU/ml

D

VRAR

Log_{10} CFU/ml
Fig. 4
Fig. 5

Concentration (ug/ml)

% inhibition of proliferation

MMA HeLa
MMA L929
succynil MA L929
Macrolactin A L929