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The Macrocyclic Peptide Antibiotic Microccin P_1 Is Secreted by the Food-Borne Bacterium *Staphylococcus equorum* WS 2733 and Inhibits *Listeria monocytogenes* on Soft Cheese

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Staphylococcus equorum **WS 2733 was found to produce a substance exhibiting a bacteriostatic effect on a variety of gram-positive bacteria. The metabolite was purified to homogeneity by ammonium sulfate precipitation and semipreparative reversed-phase high-performance liquid chromatography. Electrospray mass spectrometry confirmed the high purity of the compound and revealed a molecular mass of 1,143 Da. By two-dimensional nuclear magnetic resonance spectroscopy the substance was identified as microccin P_1 which is a macrocyclic peptide antibiotic that has not yet been reported for the genus *Staphylococcus*. A total of 95 out of 95 *Listeria* strains and 130 out of 135 other gram-positive bacteria were inhibited by this substance, while none of 37 gram-negative bacteria were affected. The antilisterial potential of this food-grade strain as a protective starter culture was evaluated by its in situ application in cheese-ripening experiments under laboratory conditions. A remarkable growth reduction of *Listeria monocytogenes* could be achieved compared to control cheese ripened with a nonbacteriocinogenic type strain of *Staphylococcus equorum*. In order to prove that inhibition was due to microccin P_1, a microccin-deficient mutant was constructed which did not inhibit *L. monocytogenes* in cheese-ripening experiments.**

For production of high-quality "red-smear cheese," complex, undefined consortia composed of many species of bacteria and yeast from different genera are often used (53). Most species of these consortia belong to the "coryneform" bacteria, but many isolates have not been classified. Since contamination of red smear cheese with *Listeria monocytogenes* has caused considerable problems, several studies have been performed on the antilisterial effects of coryneform bacteria (11, 21, 30, 40, 50). Only one bacteriocin produced by *Brevibacterium linens* has been characterized at the molecular level so far (51, 52). This strain has also been evaluated with respect to its antilisterial potential in situ on model soft cheese under laboratory conditions (15). The bacteriolytic substance linenscin produced by *B. linens* OC2 was found to inhibit *L. monocytogenes*, but it was not classified as a bacteriocin (28) and its structure has never been published.

A smaller fraction of the red-smear cheese bacteria belong to genera other than coryneform bacteria, such as *Staphylococcus* or *Micrococcus*. By screening the surface ripening flora of German and French smeared cheeses with respect to their antilisterial potential, the highly inhibitory *Staphylococcus equorum* was isolated from the surface of a traditional French Raclette cheese, where it was found in cell numbers up to 10^8 per cm^2 (the present study).

The genus *Staphylococcus* is known to produce many antibacterial substances (41, 42). According to Klaenhammer (27), these can be classified into four main groups: Lantibiotics (class I), low-molecular-weight antibiotic-like substances (class II), and high-molecular-weight (100,000 to 500,000 Da) bacteriocins (classes III and IV). Staphylococcins are class III bacteriocins in the classical sense that are sensitive to proteolytic enzymes, often being relatively heat stable (15 min, 120°C). In some cases they were found to be lipoprotein-carbohydrate complexes (class IV, e.g., staphylococcin 1580). Lantibiotics, such as gallidermin, epidermin, Pep5, epicidin, or epilancin, are low-molecular-weight polypeptide antibiotics which are characterized by the presence of unusual amino acids (lanthionine, β-methyllanthionine, forming intramolecular thioether bridges) and a high content of unsaturated amino acids (dehydroalanine and dehydrobutyrine [23, 24, 26, 44]). Class I, III, and IV molecules are ribosomally synthesized as precursor peptides which are sometimes posttranslationally modified and processed (26). In the genus *Staphylococcus*, some low-molecular-mass (1,200 to 1,400 Da), heat-stable, but non-lanthionine-containing antimicrobial peptides were found (class II), but their structure and their mode of biosynthesis is thus far unknown (7).

In this study *S. equorum* WS 2733 was shown to produce microccin P_1, which is an acceptor-site-specific inhibitor of ribosomal protein biosynthesis (12, 35) reported for the first time in the genus *Staphylococcus*. A potential application of *S. equorum* as a protective starter culture was evaluated in ripening experiments performed with model soft cheese artificially contaminated with *L. monocytogenes*.

MATERIALS AND METHODS

Bacterial strains, media, and bacteriocin assay. The microccin P_1 producer strain *S. equorum* WS 2733 was isolated from French Raclette cheese using the HGMF (hydrophobic grid membrane filter) method described previously (11). About 20 cm^2 (2 to 3 g) of the cheese surface was scraped off aseptically, homogenized in 100 ml of sterile Peptone-Tween diluent (containing 1.0 g of Peptone and 10.0 g of Tween 80 per liter) and filtered through HGMF membranes filter (QA Life Sciences, Inc., San Diego, Calif.). HGMF membranes were transferred grid-side-up to plate count agar supplemented with 3% NaCl (PC3+). After incubation at 30°C for 3 days, the membranes were transferred to

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TABLE 1. Inhibitory spectrum of microccocin P$_1$[a]

Species	No. of strains	
	Sensitive	Nonsensitive
Gram-positive bacteria		
Staphylococcus spp.		
S. aureus	15	0
S. epidermidis	2	0
S. haemolyticus	1	0
S. caseolyticus	1	0
S. xylosus	1	0
S. saprophyticus	1	0
Enterococcus spp.		
E. faecalis	1	0
E. faecium	1	0
Listeria		
L. monocytogenes	85	0
L. innocua	5	0
L. ivanovii	2	0
L. seeligeri	2	0
L. welshimeri	1	0
Bacillus cereus	7	0
	4	7
Clostridium perfringens	8	0
Lactobacillus sp.	12	0
Coryneform bacteria		0
Brevibacterium sp.	24	0
Arthrobacter sp.	30	1
Corynebacterium sp.	14	0
Microbacterium sp.	4	0
Micrococcus sp.	4	0
Gram-negative bacteria		
Salmonella sp.	0	11
Citrobacter freundii	0	5
Enterobacter sp.	0	5
Escherichia coli	0	9
Yersinia enterocolitica	0	3
Pseudomonas sp.	0	4

[a] The microccocin P$_1$-containing sterile-filtered culture supernatant (12,800 AU/ml) was used for sensitivity screening.

plates of tryptose soft agar (TB; Merck, Darmstadt, Germany) with 8 g of agar/liter inoculated with 100 μl of a 24-h culture of the indicator strain *Listeria ivanovii* (WSLC 3061) to detect inhibitory effects. Colonies corresponding to inhibition zones were picked from the membranes and purified.

Microccocin P$_1$-deficient mutants were obtained by insertional mutagenesis. The putative microccocin synthetase gene has been disrupted by integration of a tetracycline resistance cassette via homologous recombination (M. C. Carnio, K. P. Francis, and S. Scherer, submitted for publication). The wild-type strain, the microccocin P$_1$-deficient mutants, and the *S. equorum* type strain DSMZ 20674 were grown on slants of plate count agar (Merck) at 30°C and stored at 4°C. For individual experiments, the cells were subcultured in brain heart infusion broth (BHI; Merck) at 30°C.

Antibacterial activity was determined by the critical dilution method according to the method of Barefoot and Klaenhammer (2) and expressed in arbitrary units (AU) per milliliter. Serial twofold dilutions of sterile-filtered samples in 30 mM sodium phosphate buffer (pH 7.0) were spotted onto tryptose soft agar plates (TB) with 8 g of agar/liter inoculated with 100 μl of an overnight culture of the indicator strain *L. ivanovii* WSLC 3061 and incubated at 30°C for 24 h (spot-on-the-lawn assay). The titer is defined as the reciprocal of the highest dilution exhibiting complete and clear zones of inhibition on the indicator lawn. *L. ivanovii* WSLC 3061 and *L. monocytogenes* WSLC 1364 (Weihenstephan *Listeria* collection) were grown as described by Valdés-Stauber et al. (50).

For sensitivity screening performed with the sterile-filtered culture supernatant, we used a variety of bacterial strains from the Weihenstephan Culture Collection (WS) housed at the Institute of Microbiology, Forschungszentrum für Milch und Lebensmittel, Weihenstephan, Freising, Germany.

Isolation and purification of microccocin P$_1$. Microccocin P$_1$ was purified from 20-liter cultures of *S. equorum* WS 2733 grown at 30°C in BHI broth (Merck) for 24 h. The cells were pelleted by centrifugation (12,000 × *g*, 20 min, 4°C). The antibiotic present in the culture supernatant was concentrated by ammonium sulfate precipitation. The pellet was resuspended in 650 ml of sodium phosphate

buffer (50 mM, pH 7.0) and applied to a reversed-phase column (Pharmacia XK 16/70; 700 by 16 mm, silica gel 100 C$_{18}$, 40 to 63 μm; Fluka) equilibrated with buffer A (0.1% [vol/vol] trifluoroacetic acid [TFA] H$_2$O) at a flow rate of 1 ml/min. After a washing with 240 ml of buffer A the column was eluted at a flow rate of 4 ml/min with increasing amounts of buffer B (80% acetonitrile–20% H$_2$O–0.1% TFA [vol/vol/vol]). The antibiotic-containing fractions were collected, pooled, and redissolved in 50% ethanol (fraction II). Final purification was achieved by reversed-phase high-performance liquid chromatography (RP-HPLC) on an RP column (Eurospher 100-C$_{18}$, 7 μm, 250 by 8 mm; Knauer, Berlin, Germany) equilibrated with buffer A. Microccocin P$_1$ was eluted by a linear gradient of 0 to 80% buffer B in 30 min at a flow rate of 3.5 ml/min. All purification steps were performed at room temperature. The chromatographic equipment was obtained from Pharmacia-LKB (Gradi-Frac System; pump, P-50; detectors, Uvicord SII and VWM 2141; detection wavelengths, 280 and 220 nm, respectively) and Perkin-Elmer (Liquid Chromatograph Series 400). In order to monitor purity, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a discontinuous 8 to 18% gradient gel (Excel Gel; Pharmacia-LKB). One-half of the gel was silver stained according to the method of Blum et al. (5), while the other half was assayed directly for antimicrobial activity by the modified test described by Bhunia et al. (4).

GC-MS analysis. The RP-HPLC-purified sample was hydrolyzed under N$_2$ atmosphere in 6 N HCl at 110°C for 24 h. The hydrolyzate was derivatized to *n*-propyl esters with 3 N HCl in *n*-propanol (110°C, 10 min). After completion of the reaction the reagents were removed with N$_2$. Reaction with trifluoroacetic anhydride (110°C, 10 min) subsequently gave the trifluoroacetamides. After removal of the reagents with N$_2$, the sample was dissolved in toluene and analyzed on a Carlo-Erba 2900 gas chromatograph (GC). The separation was performed on a fused-silica capillary (20 by 0.25 mm, inner diameter; $d_f \approx 0.13$ μm) coated with Chirasil-γ-Dex (19) as stationary chiral phase. The GC was coupled to a MAT 112S mass spectrometer (MS; Finnigan, Bremen, Germany) with an AMD Intectra Data System. For electron-impact ionization, an energy of 70 eV was used.

MS and NMR spectroscopy. MS analysis was performed with positive-ion-mode detection on a Apex II-ESI-FT-ICR MS (Bruker Daltonics, Billerica, Mass.) operating at 4.7 T. One- and two-dimensional nuclear magnetic resonance (NMR) experiments were performed on a AMX2-600 spectrometer (Bruker, Karlsruhe, Germany) operating at a proton frequency of 600.13 MHz and equipped with a 5-mm inverse triple resonance probehead with a self-shielded Z-gradient coil. The following experiments were recorded at 298 K using a solution of 1.4 mg of the purified sample dissolved in 0.5 ml CD$_3$OH: ^{1}H NMR, TOCSY (total correlation spectroscopy), NOESY (nuclear overhauser spectroscopy), and HSQC (heteronuclear single quantum coherence). For suppression of the hydroxy resonance of the solvent a Watergate sequence was appended to the homonuclear experiments. An HMBC (heteronuclear multiple
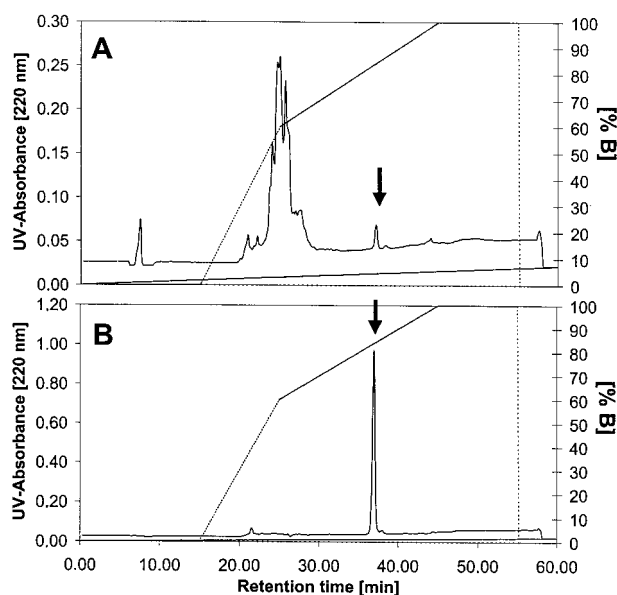

FIG. 1. Elution profile of microccocin P$_1$ (A) RP-HPLC on a Eurosphor 100-C$_{18}$ column (250 by 8 mm; sample volume, 10 μl; flow rate, 3.5 ml/min; linear acetonitrile gradient, 0 to 80% in 30 min) of crude concentrate (fraction II). All contaminating compounds were separated at the beginning of the linear gradient (0 to 60% B). (B) Analytical separation of pooled fraction IV. The pure antibacterial compound was eluted as a distinct, symmetrical peak at about 84% of solvent B (67% acetonitrile). The arrow indicates the activity.
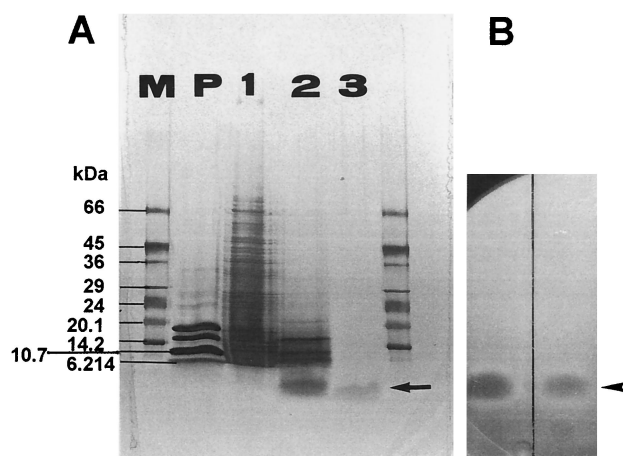
FIG. 2. SDS-PAGE of preparations after various stages of purification. (A) Silver-stained Excel Gel (gradient, 8 to 18% T; Pharmacia-LKB). Lane 1, crude concentrate after ammonium sulfate precipitation; lane 2, fraction after preparative RP-LC; lane 3, purified peptide after RP-HPLC; M, molecular weight marker SDS-7L (Sigma); P, peptide length standard (Pharmacia-LKB). (B) Direct detection of antilisterial activity on the gel overlaid with tryptose soft agar seeded with the indicator strain *L. ivanovii* WSLC 3061. The inhibition zones after incubation overnight at 30°C correspond to the silver-stained bands in Fig. 2A, lanes 2 and 3.

bond correlation) spectrum was acquired at 318 K using a solution of 6 mg of the purified sample dissolved in 0.5 ml of CD_3OH. HSQC and HMBC experiments were performed with gradient selection. A ^{13}C NMR spectrum of the second sample was recorded on a AC250 spectrometer (Bruker, Karlsruhe, Germany) operating at a carbon frequency of 62.90 MHz and equipped with a 5-mm dual-probe head. Chemical shifts (see Table 2) were referenced to the signals of the solvent at $\delta(^1H) = 3.35$ ppm and $\delta(^{13}C) = 49.0$ ppm.

Cheese-ripening experiments. To evaluate the antilisterial potential of the *S. equorum* strain WS 2733 in situ on soft cheese, model ripening with defined single-strain cultures was performed according to the method of Eppert et al. (15) under laboratory conditions in glass desiccators. Micrococcin-deficient (*mic*) mutants and the nonbacteriocinogenic (Bac⁻) *S. equorum* type strain DSMZ 20674 were used as negative controls. Unripened soft cheese of the type "Weinkäse" were obtained from a local dairy after salting and being hand-smeared in petri dishes using gloves and 50 ml of a brine solution with 5% NaCl inoculated with the test strain and the control strains, respectively. The first day of smearing was designated day 1 of the ripening period. Smearing was applied five times (days 1, 3, 5, 7, and 10) at intervals of 2 or 3 days. The cheese was artificially contaminated with *L. monocytogenes* WSLC 1364 (from the Vacherin Mont d'Or outbreak) on day 1 of smearing. The contamination levels were 4.5×10^4 CFU/ml of brine (resulting in 1.5×10^2 CFU/cm^2 of cheese surface), 1.5×10^3 CFU/ml of brine (10 CFU/cm^2), and 2.0×10^2 CFU/ml of brine, respectively. The bacterial counts of the *Staphylococcus* ripening culture in the smear brine were 1.3×10^8/ml (DSMZ 20674), 1.5×10^8/ml (*mic* mutant), and $1.8 \times 10/$ml (WS 2733), respectively. In order to cope with a potentially nonuniform distribution of listeriae on the cheese surface at low contamination levels, a large part of the surface was investigated for cell counts (see below).

For the determination of bacterial cell counts, one cheese for each storage time was examined. First, 20 g of the cheese surface (45 cm^2) was homogenized in 180 ml of 1.75% trisodium citrate-dihydrate solution using a stomacher. Serial decimal dilution series on these suspensions were plated on plate count agar supplemented with 3% NaCl (PC3+) for aerobic plate counts (30°C, 48 h) and on Oxford agar (Oxoid, Hampshire, England) for listerial counts (37°C, 48 h). Then, 0.1 ml of each dilution was plated in duplicate except for the early stage of the ripening period, at which 1 ml of the initial suspension (dilution 10^{-1}) was
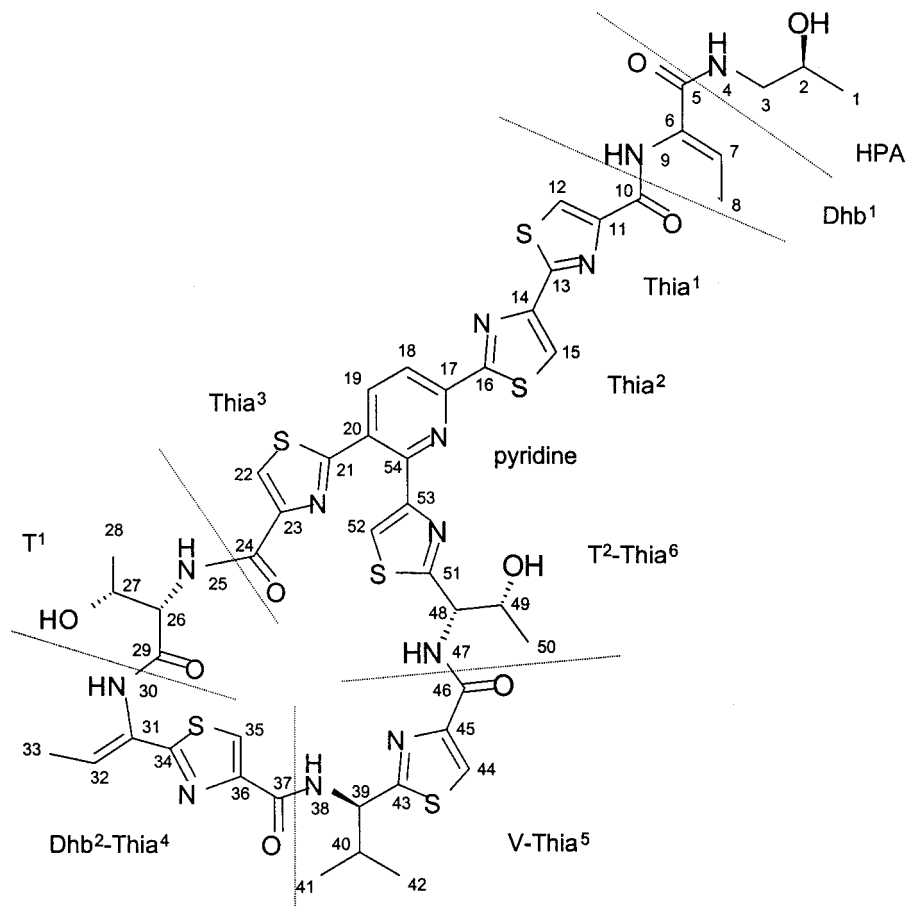

FIG. 3. Structure of micrococcin P_1. Structural units separated by peptide bonds are divided by dashed lines.

distributed on 10 Oxford agar plates. The cell counts were calculated per square centimeter. The selective enrichment procedure for detection of low levels of *L. monocytogenes* was performed according to the international IDF standard 143A:1995. A total of 25 g (equivalent to 45 cm²) of the cheese surface was blended with 225 ml of Tryptone soy yeast extract broth using a peristaltic blender. After incubation at 30°C for 48 h, a loopful of the enrichment culture was streaked onto Oxford agar.

RESULTS

Isolation of *S. equorum* WS 2733 and inhibitory spectrum. An *S. equorum* strain, inhibiting a variety of *L. monocytogenes* strains, was isolated from the surface of a French Raclette cheese. This bacterial consortium was composed of approximately 7.8×10^8 CFU/cm² (18.5%) orange, 4.4×10^8 CFU/cm² (10.4%) yellow pigmented, and 2.9×10^9 CFU/cm² (68.7%) white coryneform bacteria partly identified as *B. linens* and *Corynebacterium fascians*. The fraction of the *S. equorum* strain was 2.5% (1.0×10^8 CFU/cm²) of the total aerobic plate count (4.2×10^9 CFU/cm²). The peptide antibiotic present in the culture supernatant showed a wide spectrum of inhibitory activity that was not restricted to closely related species (Table 1). A total of 95 out of 95 *Listeria* strains and 130 out of 138 other gram-positive bacteria were inhibited. Nearly all gram-positive bacteria tested were sensitive, whereas no inhibition of 37 gram-negative bacteria was observed. The antimicrobial peptide revealed to be highly inhibitory against all tested *L. monocytogenes* strains, while only 36% of the tested *Bacillus cereus* strains were sensitive. The mode of action in the tested concentrations (12,800 AU/ml) was bacteriostatic (data not shown).

Purification of the active substance. Purification of microccin P_1 was achieved by RP chromatography. The final polishing step (RP-HPLC) resulted in a 1,600-fold increase in specific activity with 25% recovery. The elution profile from the RP-C_{18} column is presented in Fig. 1. The antimicrobial substance was eluted at about 84% buffer B (67% acetonitrile). The strong binding of the compound to the C_{18} column at relatively high acetonitrile concentrations was indicative of a highly hydrophobic molecule. The purification procedure was followed by SDS-PAGE analysis. A single band was detected migrating at an M_r of <2,500 (Fig. 2A, lane 3), which is consistent with a pure preparation and indicative of a relatively small compound. The substance was localized in situ by its antibacterial activity. A clear and distinct zone of inhibition (Fig. 2B), corresponding to an M_r of <2,500 was revealed on the gel overlaid with a *L. ivanovii* WSLC 3061 indicator lawn.

Structure. MS analysis confirmed the purity of the sample and revealed a molecular mass of 1,143 Da for the substance. ^1H and ^{13}C NMR spectra (data not shown) comprised signals for 46 hydrogens (hydrogens of the three hydroxy groups exchanged fast with the solvent) and 48 carbon atoms. Considering the molecular mass, the moderate number of carbon and hydrogen atoms in the molecule indicated a high content of heteroatoms. The spin systems of one valine (Fig. 3, V), two threonine (T), and two didehydrobutyrine (Dhb) residues, as well as of a 2-hydroxypropylamide (HPA) moiety, were traced in a TOCSY spectrum (data not shown). Sequential connectivities between HPA and Dhb¹ and between T¹ and Dhb² (Fig. 3), respectively, were determined by a NOESY experiment by which also the Z-configuration of both Dhb residues was confirmed. The HSQC spectrum contained cross-peaks for eight (hetero)aromatic methine units. Heteronuclear one-bond coupling constants of $^1J_{CH} = 196$ Hz which were observed in the HMBC spectrum for six of the respective methine units suggested the presence of six thiazole rings in the molecule. Methods for detection of thiazole containing

TABLE 2. ^1H and ^{13}C chemical shifts observed for microccin P_1 in CD_3OH at 318 K

No.[a]	Chemical shift (ppm)		No.[a]	Chemical shift (ppm)	
	δ(^1H)	δ(^{13}C)		δ(^1H)	δ(^{13}C)
1	1.19	20.6	28	1.55	20.8
2	3.95	67.5	29		170.9
3	3.37, 3.25	48.3	30	9.53	
4	7.91		31		130.6
5		167.4	32	6.57	130.9
6		131.2	33	1.86	14.1
7	6.73	131.8	34		168.0
8	1.85	13.7	35	8.10	125.1
9	9.57		36		149.7
10		162.1	37		162.5
11		151.1	38	8.48	
12	8.32	126.3	39	5.24	57.4
13		163.9	40	2.61	34.4
14		150.9	41	1.14	19.2
15	8.33	122.1	42	1.00	20.0
16		169.9	43		171.5
17		151.6	44	8.26	125.6
18	8.32	119.9	45		150.4
19	8.28	141.1	46		163.1
20		129.9	47	8.50	
21		166.1	48	5.26	57.5
22	8.20	126.3	49	4.31	69.2
23		151.0	50	1.18	20.5
24		162.5	51		172.2
25	7.94	–	52	7.95	121.8
26	4.79	59.3	53		155.0
27	4.60	69.6	54		152.7

[a] Numbers correspond to those presented in Fig. 3.

amino acids were described for the microcin B17 structure elucidation (3).

The GC-MS analysis of the acid hydrolyzate (data not shown) accounted for one mole each of 1-amino-2-propanol, L-threonine, and 2-(1-amino-2-methylpropyl)-thiazole-4-carboxylic acid and reflected the structural units HPA, T¹, and V-Thia⁵.

These data strongly suggested a close relationship or identity to microccin P_1 (9). The two-dimensional identity of the substance isolated by us with microccin P_1 (Fig. 3) was proven by the complete structure elucidation of the molecule relying mainly on HMBC connectivities. Thereby, a detailed assignment of the ^1H and ^{13}C chemical shifts was obtained (Table 2), which has never been published for microccin P_1. Apart from C-26 and C-27 of residue T¹, the absolute configurations of the sterocenters were not determined experimentally but were based on biosynthetic considerations and assumed to be as reported for microccin P_1.

Growth reduction of *L. monocytogenes* on cheese surface. The development of the pH and total aerobic plate counts during the ripening process are shown in Fig. 4A and can be considered typical for the ripening of industrial red-smear cheese using single-strain cultures (15). The total aerobic plates counts of the cheese surface before smearing were about 2.0×10^6 CFU/cm², and the pH values were in the range of 5.0. At the end of the ripening process all cheeses reached total aerobic cell counts of approximately 6.0×10^8 CFU/cm². A growth reduction of *L. monocytogenes* on cheese ripened with the microccin P_1-producing (Mic⁺) *S. equorum* wild-type strain WS 2733 was observed when compared to control cheese ripened with the bacteriocin-negative strain DSMZ 20674 and
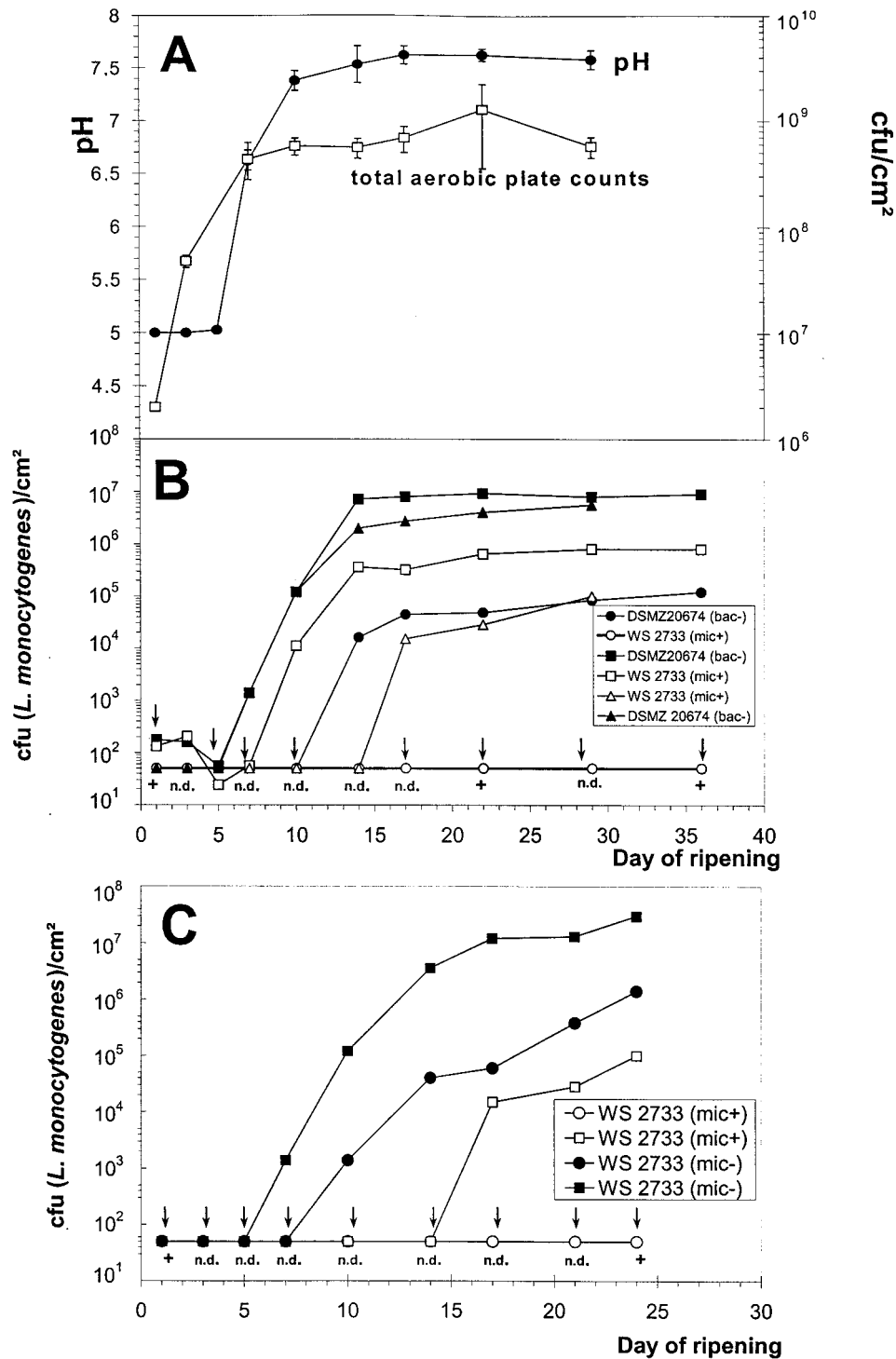
FIG. 4. Cheese ripening with defined single-strain cultures of *S. equorum* WS 2733. (A) Development of pH on the cheese surface and aerobic plate counts (PC3+ agar). (B) Growth reduction of *L. monocytogenes* WSLC 1364. *Listeria* cell counts on the cheese surface (Oxford agar) after contamination at day 1 with 10^4 CFU/ml (10^2 CFU/cm^2) (■ and □), 10^3 CFU/ml (10 CFU/cm^2) (△ and ▲), and 10^2 CFU/ml (● and ○), are shown. Control cheeses were ripened with the micrococcin-negative type strain DSMZ 20674 (bac−), closed symbols. (C) *Listeria* cell counts on the cheese surface (Oxford agar) after contamination at day 1 with 10^3 CFU/ml (■ and □) and 10^2 CFU/ml (10 CFU/cm^2) (● and ○). Control cheeses were ripened with the micrococcin-deficient mutant (mic−), closed symbols. The micrococcin-producing wild-type strain (mic+) is represented by open symbols. Arrows indicate that even by using enrichment procedures, listeriae could not be detected (n.d.). The measuring points mark the detection limit (50 to 100 CFU/cm^2) of the direct plating method. +, Listeriae could be detected only after enrichment procedures.

the micrococcin-deficient mutant (Mic$^-$). The effect was dependent on the contamination level. When challenged with 10^4 CFU/ml of brine (10^2 CFU/cm^2), a reduction of the viable cell counts by more than 1 log unit could be observed, reaching a final level of 8.0×10^5 CFU/cm^2, while listeriae on control cheese grew to 9.0×10^6 CFU/cm^2. When challenged with 10^3 CFU/ml of brine, a growth reduction of 1 to 2 log cycles could be demonstrated. Remarkable effects could be achieved with low contamination levels (10^2 CFU/ml of brine). The *Listeria* cells grew to approximately 1.2×10^5 CFU/cm^2 on the control cheese ripened with the micrococcin-negative strain DSMZ 20674 (Bac$^-$) and to 1.5×10^6 CFU/cm^2 on control cheese ripened with the micrococcin-deficient knockout mutant (Mic$^-$), whereas on cheese inoculated with the wild-type *S. equorum* WS 2733 the growth of listeriae was completely inhibited.

DISCUSSION

Micrococcin P$_1$ (Fig. 3) is a macrocyclic peptide antibiotic of the thiocillin-thiazolyl class (8, 9, 13, 25, 32, 54). Recently, a complete synthesis was published by Okumura et al. (34). The peptide binds to complexes formed between the L11 protein of the 50S ribosomal subunit and a nucleotide sequence within the 23S rRNA and inhibits the elongation factor EFG-dependent translocation step of the growing peptide chain. Resistant mutants of *B. subtilis* and *B. megaterium* either possess a structurally altered 50S subunit or are specifically methylated at a single site (nucleotide A-1067) of the 23S rRNA (39, 45, 46). It has been demonstrated that this macrocyclic thiopeptide antibiotic is synthesized nonribosomally and involves a multifunctional peptide synthetase, which was designated micrococcin synthase (Carnio et al., submitted).

So far, micrococcin P$_1$ has been purified from a bacterium related to *Micrococcus varians* (20, 48) and from *Bacillus pumilus* (1, 16), isolated from sewage and soil, respectively. Breiter et al. (7) have reported antibiotically active substances excreted by seven strains belonging to *Staphylococcus* and *Micrococcus* species isolated from animal sources (partridge, pig, and dog). Two basic substances were isolated and designated micrococcin M1 and M3. The physicochemical and spectroscopic data, as well as investigations of hydrolysis products, indicated a close relationship to micrococcin P$_1$. However, the structure of these compounds has never been reported. The antibacterial substance secreted by *S. equorum* WS 2733 was identified as micrococcin P$_1$ by spectroscopic evidence. Based on biosynthetic considerations, the absolute configuration of the stereocenters was assumed to be as reported for micrococcin P$_1$. In this context it is of interest that other thiazole- and oxazole-containing polypeptides such as the bacteriocin microcin B17 originate from ribosomally synthesized precursors which are posttranslationally modified (3).

S. equorum was revealed to be a potent inhibitor of growth of *L. monocytogenes* on the cheese surface. The phenomenon of complete inhibition of listeriae occurs only when the inhibitory strain is used as sole starter and when contamination with *Listeria* sp. is performed in the early stage of ripening at a low concentration level (10^2 CFU/ml of brine), which is a high titer for contamination occurring in the dairy industry. This is in accordance with other studies reporting on the inhibition of *Listeria* sp. on cheese surfaces (9, 14, 15, 17, 18, 29, 33, 36, 47, 49, 51, 55). The limited effects of antilisterial bacteria clearly show that there is a need for developing hurdle concepts in the dairy industry in which bacteriocinogenic bacteria play a certain role. However, such protective starters cannot replace a good hygienic practice during food processing.

Although micrococcin P$_1$ exhibits a broad activity spectrum, inhibiting nearly all gram-positive bacteria when tested on agar plates (spot-on-the-lawn-assay; Table 1), the antibiotic allows the formation of a stable ecological system on the surface of the French Raclette cheese. However, when combined with other surface-ripening cultures in cheese-ripening experiments under laboratory conditions, *S. equorum* WS 2733 dominated the ripening flora by suppressing the other gram-positive coryneform bacteria (data not shown). This finding may be explained by the different cheese types: on the surface of a soft cheese the development of the ripening microflora may be completely different from the one on the Raclette surface. It appears, however, to be more likely that the bacterial consortium growing on the surface of the French Raclette cheese constitutes a well-balanced, stable ecosystem which has developed over decades of cheese production. Biocontrol of listeriae on soft cheese by protective ripening bacteria will therefore be more complicated than just adding an antilisterial strain to a well-established smear-cheese flora.

S. equorum was originally isolated from the skin of healthy horses and was first described by Schleifer et al. (43), but it has often been associated with food products. For instance, together with *Staphylococcus xylosus*, it constitutes the predominant organism present during ripening of an Iberian dry cured ham (37), contributing to the characteristic flavor of the final product. It is also part of the ripening flora from traditional French cheeses (22) and was isolated from goat milk and cheese (31). Moreover, 5 to 15% of the total cell counts of the microflora of the German Tilsit cheese consist of staphylococci classified as *S. equorum* (6). This species belongs to the group of coagulase-negative staphylococci and has never been reported to be involved in diseases. There would, therefore, be good reason to consider *S. equorum* a GRAS (generally recognized as safe) organism. In the case of our isolate, however, it should be noted that micrococcin P$_1$ has to be classified as an antibiotic. With respect to its potential pharmaceutical use it may, therefore, be wise to be careful in spreading this strain widely in the human community before its pharmaceutical potential is evaluated.

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