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Antifungal 3-Hydroxy Fatty Acids from *Lactobacillus plantarum* MiLAB 14

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We report the identification and chemical characterization of four antifungal substances, 3-(*R*)-hydroxydecanoic acid, 3-hydroxy-5-*cis*-dodecenoic acid, 3-(*R*)-hydroxydodecanoic acid and 3-(*R*)-hydroxytetradecanoic acid, from *Lactobacillus plantarum* MiLAB 14. The concentrations of the 3-hydroxy fatty acids in the supernatant followed the bacterial growth. Racemic mixtures of the saturated 3-hydroxy fatty acids showed antifungal activity against different molds and yeasts with MICs between 10 and 100 μg ml^{-1}.

Lactic acid bacteria (LAB) have a long history of use as biopreservatives for food and feed storage. The general preserving ability of lactic acid and other fermentation end products and the antibacterial effects of LAB proteinaceous bacteriocins are well documented (14, 24). Recent research has revealed that LAB can produce low-molecular-weight antifungal substances, e.g., phenyllactic acid, *p*-hydroxyphenyllactic acid (12, 25), cyclic dipeptides such as cyclo(Gly-L-Leu), cyclo(L-Phe-L-Pro), and cyclo(L-Phe-*trans*-4-OH-L-Pro) (18, 25), benzoic acid, methylhydantoin, mevalonolactone (18), and short-chain fatty acids (FAs) (4).

Previously, a large number of LAB strains with antifungal effects have been isolated in vitro from plant material stored under anaerobic conditions (16). In a continuous study of LAB strains with antifungal effects, procedures for isolating antifungal compounds among a background of high concentrations of lactic acid were devised (25). In this study, the characterization of four antifungal 3-hydroxy FAs (3-OH-FAs) from *Lactobacillus plantarum* MiLAB 14 is reported.

Strain MiLAB 14, isolated from lilac flowers (16), was identified as *L. plantarum* from both the fermentation pattern and the 16S rRNA gene sequence. The API 50 CHL test (bioMérieux, Marcy L'Etoile, France) was used for identification by fermentation pattern. Chromosomal DNA isolation and PCR amplification were performed as previously described (25). Approximately 1,400 bp of the 16S rRNA gene were sequenced as previously described (25) but with additional customized primers covering the whole fragment.

The strain MiLAB 14 was grown on MRS agar and stored as previously described (15). The molds *Aspergillus fumigatus* J9, *Aspergillus nidulans* J283 (FSGC A4 wt), *Penicillium roqueforti* J268 (IBT 6754), and *Penicillium commune* J238 (IBT 12400) and the yeasts *Kluyveromyces marxianus* J137 (CBS 6556), *Pichia anomala* J121, and *Rhodotorula mucilaginosa* J350 (CFSQE 63) were used as target organisms for assay of antifungal activity. The target fungi were chosen to represent potential spoilage fungi in silage and dairy products (20). All fungi are kept in the culture collection at the Department of Microbiology, Swedish University of Agricultural Sciences. Molds and yeasts were prepared as previously described (15). A microtiter plate assay (15) was used for bioassay-guided fractionation. *A. fumigatus* was used as the target organism, as it has been shown to be sensitive to antifungal strains of LAB (16) as well as being a serious pathogen of animals and humans (6).

The cell-free supernatant of *L. plantarum* MiLAB 14 from a 48-h still culture at 30°C was obtained and fractionated by the same method with solid-phase extraction (SPE) and high-performance liquid chromatography, as previously described (25). As a negative control, noninoculated MRS broth was fractionated and evaluated in the bioassay by the procedure used for the cell-free supernatant. The structures of the antifungal compounds were determined by nuclear magnetic resonance (NMR) spectroscopy, electrospray ionization mass spectrometry (ESI-MS), and gas chromatography-mass spectrometry (GC-MS). Absolute configuration was determined by preparation of 3-*O*-methyl *N*-(*S*)-phenylethylamide derivates of the 3-OH-FAs followed by GC-MS analysis (7).

Three aliquots of 800-ml cultures in MRS broth were inoculated with 10^4 bacteria ml^{-1}, and concentrations of 3-OH-FAs, growth (number of CFU on MRS agar plates), and pH were monitored for 78 h. Each sample of cell-free supernatant (10 ml) was fractionated by SPE (Isolute, C_{18} end capped, 1 g). The fraction eluted with 4 ml of aqueous 95% acetonitrile, after a wash with 3 ml of aqueous 30% acetonitrile, was dried under vacuum. The material was dissolved in 100 μl of hexane in 1.5-ml Eppendorf tubes and derivatized with 50 μl of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (Supelco, Stainheim, Germany) and 10 μl of pyridine at 80°C for 1 h. GC-MS was performed on a fused-silica capillary column using a temperature gradient (70°C for 3 min; 70 to 240°C at 10°C min^{-1}; injector, 240°C; interface, 260°C; carrier gas, He, 1 ml min^{-1}). The reference was a mixture of trimethylsilyl derivatives of 3-hydroxydecanoic acid, 3-hydroxydodecanoic acid, and 3-hydroxytetradecanoic acid. The concentrations of the 3-OH-FAs were determined by using 3-hydroxyundecanoic acid as an in-

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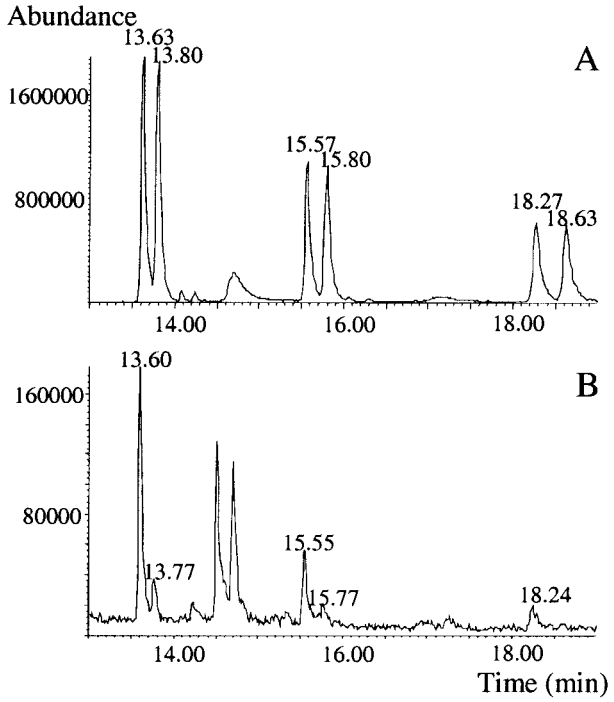

FIG. 1. Extracted ion chromatogram of *m/z* 120 for 3-*O*-methyl *N*-(*S*)-phenylethylamide derivatives of a standard solution with racemic 3-hydroxydecanoic acid (R = 13.63 min and S = 13.80 min), 3-hydroxydodecanoic acid (R = 15.57 min and S = 15.80 min), and 3-hydroxytetradecanoic acid (R = 18.27 min and S = 18.63 min) (A) and samples from *L. plantarum* MiLAB 14 after SPE of cell-free supernatant, 3-hydroxydecanoic acid (R = 13.60 min and S = 13.77 min [the (S) form is formed by racemization of the sample during derivatization]), 3-hydroxydodecanoic acid (R = 15.55 min and S = 15.77 min [the (S) form is formed by racemization of the sample during derivatization]), and 3-hydroxytetradecanoic acid (R = 18.24 min) (B) (see the text for conditions).

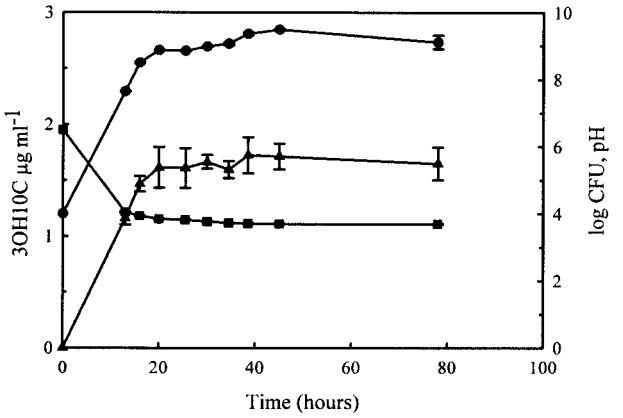

FIG. 2. Concentration of 3-hydroxydecanoic acid (▲), number of CFU (●), and pH (■) during still-culture growth of *L. plantarum* MiLAB 14 in MRS broth at 30°C. Bars indicate standard deviations (n = 3).

ternal standard (2 μg ml of supernatant^{-1} added before preparation of cell-free supernatant) and assuming similar response factors for the different 3-OH-FAs.

The MIC was determined in duplicate as the lowest concentration where total inhibition of spore germination was observed. MICs were determined for decanoic acid, 2-hydroxydodecanoic acid, 3-hydroxydecanoic acid, 3-hydroxyundecanoic acid, 3-hydroxydodecanoic acid, and 3-hydroxytetradecanoic acid (Larodan Fine Chemicals, Malmö, Sweden), and all OH-FAs were racemic. FAs were dissolved in methanol and diluted with 10 mM acetic acid. The molds *A. fumigatus*, *A. nidulans*, *P. roqueforti*, and *P. commune* and the yeasts *K. marxianus*, *P. anomala*, and *R. mucilaginosa* were used as target organisms. MIC determinations were performed as serial twofold dilutions by a microtiter plate method (15) with malt extract broth (2%) instead of MRS.

Two active compounds, 3-hydroxydecanoic acid and 3-hydroxy-5-*cis*-dodecenoic acid, were isolated from cell-free supernatant by bioassay-guided fractionation. The former compound was identified by comparing data from NMR, ESI-MS, and high-performance liquid chromatography with data from commercial racemic 3-hydroxydecanoic acid, whereas the latter compound was identified by comparison of experimental and literature NMR data (9, 11) together with the molecular mass from ESI-MS. No activity was observed from the corresponding fractions isolated from noninoculated MRS broth. Further, two active compounds, 3-hydroxydodecanoic acid and 3-hydroxytetradecanoic acid, were identified in cell-free supernatant by GC-MS. The saturated 3-OH-FAs were determined to have the (R) configuration (Fig. 1), whereas the absolute configuration of 3-hydroxy-5-*cis*-dodecenoic acid could not be determined due to the lack of reference compounds. The presence of small peaks corresponding to 3-OH-FAs in the (S) configuration in Fig. 1 is due to racemization during the derivatization procedure. This racemization was detected by using methanol-d_4/D_2O instead of methanol/water as the solvent in the hydrolysis step (data not shown). All saturated 3-OH-FAs (17, 19, 22) in this work as well as 3-hydroxy-5-*cis*-dodecenoic acid (3, 11) have previously been isolated. Hydroxy FAs are commonly found in animals, plants, and fungi (26), and their antifungal properties have been reported (8, 10). In bacteria, 3-OH-FAs are present in lipopolysaccharides (1) or in polyhydroxyalkanoic acids (23). On the other hand, gram-positive bacteria, such as LAB, have no lipopolysaccharides, and there are no reports on poly-hydroxyalkanoic acids from LAB. The cellular FAs that have been used for classification of different LAB are mainly saturated and monounsaturated FAs containing 12 to 20 carbons (5, 21) and representing more than 90% of all cellular FAs in LAB (5). However, Lee et al. (13) identified 2-hydroxyhexadecanoic acid and 3-hydroxyheptadecanoic acid from different *Leuconostoc* strains. LAB can metabolize unsaturated FAs to OH-FAs (27, 28), indicating metabolic pathways for hydroxylation of FAs, but the exact role of 3-OH-FAs in LAB metabolism remains to be elucidated.

The concentration of 3-(R)-hydroxydecanoic acid in the culture supernatant increased during the logarithmic growth phase of *L. plantarum* MiLAB 14 and reached a maximum of 1.7 μg ml^{-1} after 38 h (Fig. 2). The concentration of 3-(R)-hydroxydecanoic acid increased only during the exponential phase and not when the cells reached the stationary phase. This indicates that 3-OH-FAs do not originate from disrupted cell membranes of dead bacteria but instead are excreted to

TABLE 1. MIC of racemic mixtures of fatty acids for different yeasts and molds in a microtiter plate assay

Organism	MIC ($\mu g\ ml^{-1}$) of[a]:					
	C10	2-OH-C12	3-OH-C10[b]	3-OH-C11	3-OH-C12[b]	3-OHC14[b]
P. roqueforti	25	5	25	10	25	50
P. commune	50	25	100	50	50	>100[c]
A. nidulans	100	25	50	50	25	>100[c]
A. fumigatus	100	25	100	50	25	>100[c]
K. marxianus	25	25	50	25	25	10
R. mucilaginosa	5	5	10	25	10	10
P. anomala	25	25	50	25	25	50

[a] C10, decanoic acid; 2-OH-C12, 2-hydroxydecanoic acid; 3-OH-C10, 3-hydroxydecanoic acid; 3-OH-C11, 3-hydroxyundecanoic acid; 3-OH-C12, 3-hydroxydodecanoic acid; 3-OH-C14, 3-hydroxytetradecanoic acid.
[b] Detected in *L. plantarum* MiLAB 14 culture supernatant.
[c] Not determined due to low solubility.

the culture broth by living bacterial cells. The concentrations of 3-hydroxy-5-*cis*-dodecenoic acid (1.0 $\mu g\ ml^{-1}$), 3-(*R*)-hydroxydodecanoic acid (0.5 $\mu g\ ml^{-1}$), and 3-(*R*)-hydroxytetradecanoic acid (0.2 $\mu g\ ml^{-1}$) were lower than the concentration of 3-(*R*)-hydroxydecanoic acid (1.6 $\mu g\ ml^{-1}$) after 78 h of growth, but all concentrations varied over time in a similar way (data not shown). None of the acids were detected at the time of inoculation.

For both yeasts and molds, the MICs for total growth inhibition were between 10 and 100 $\mu g\ ml^{-1}$ for the racemic forms of the 3-OH-FAs (Table 1). Yeasts appeared to be more sensitive than filamentous fungi to the different 3-OH-FAs. Among the filamentous fungi, *P. roqueforti* was the most sensitive (5 to 50 $\mu g\ ml^{-1}$), whereas *A. fumigatus*, previously found to be highly sensitive to LAB strains with antifungal properties (16), was among the least sensitive fungi (25 to 100 $\mu g\ ml^{-1}$). The concentrations of the 3-OH-FAs found in MiLAB 14 supernatant are about 10 to 200 times lower than the MICs. However, the 3-OH-FAs could still contribute to the antifungal activity of *L. plantarum* MiLAB 14, since higher local concentrations of the 3-OH-FAs are expected to be found close to bacterial colonies and there could also be synergistic effects.

The mechanisms behind the antifungal effect of the 3-OH-FAs are not known, but the MICs for all FAs and target organisms are within a fairly narrow range. This suggests that all the 3-OH-FAs from *L. plantarum* MiLAB 14, as well as 2-hydroxydodecanoic acid and decanoic acid, would have similar modes of action. As enantiomerically pure 3-OH-FAs were not available, possible differences in MICs between (*R*) and (*S*) forms could not be established. One general mechanism that has been proposed for antifungal FAs is that the activity is due to detergent-like properties of the compounds, affecting the structure of cell membranes of the target organisms. Indeed, *cis*-9-heptadecenoic acid, a compound similar to the 3-OH-FAs identified here, readily partitions into the lipid bilayers of fungal membranes (2). This increases membrane permeability and the release of intracellular electrolytes and proteins and, eventually, leads to cytoplasmic disintegration of fungal cells.

Future studies on lactic acid bacteria with antifungal properties could lead to useful biopreservation systems, preventing fungal spoilage and mycotoxin formation in both food and animal feed.

Nucleotide sequence accession number. The sequence determined in this study has been deposited in GenBank with accession number AY383631.

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