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Cloning and Heterologous Expression of Hematin-Dependent Catalase Produced by *Lactobacillus plantarum* CNRZ 1228

Hikmate Abriouel,[1]† Anette Herrmann,[1] Joachim Stärke,[2] Nuha M. K. Yousif,[1] Agus Wijaya,[1] Bernhard Tauscher,[2] Wilhelm Holzapfel,[1] and Charles M. A. P. Franz[1]*

Institute of Biotechnology and Molecular Biology[1] and Institute of Biological Chemistry,[2] Federal Research Centre for Nutrition, D-76131 Karlsruhe, Germany

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Lactobacillus plantarum **CNRZ 1228 exhibited heme-dependent catalase activity under environmental conditions similar to those encountered during sausage fermentation. The 1,455-bp catalase gene (*katL*) was cloned and encoded a protein of 484 amino acids. Expression of *katL* in a heterologous host showed that *katL* encodes a functional catalase. PCR screening of selected strains of lactic acid bacteria for *katL* indicated the presence of similar genes in other strains of lactobacilli.**

Catalases are antioxidant metalloenzymes that disproportionate hydrogen peroxide to water and oxygen during aerobic growth. Moreover, when the H_2O_2 concentration is low, catalase may also catalyze the oxidation of electron donors, such as ethanol or phenols (13). Lactic acid bacteria (LAB) were generally considered catalase-devoid microorganisms until the last decade, when catalase activity was reported in *Lactobacillus*, *Pediococcus*, *Enterococcus*, and *Leuconostoc* species (3, 5, 8, 9, 11, 20, 21). There are two major classes of catalase, the true or heme-dependent catalase, and the manganese-containing catalase or pseudocatalase (8). The best-studied catalase is the heme-dependent catalase, which is induced by an exogenous heme group that is incorporated into the apoenzyme.

Hydrogen peroxide can interfere with organoleptic properties of foods and may, for example, lead to rancidity and discoloration of fermented meat products (1). The presence of catalase activity in LAB may be an important property of starter culture strains, especially in lactobacilli, which are of great commercial value for meat fermentations (10). In addition to eliminating hydrogen peroxide, catalase can also catalyze the degradation of phenolic compounds which occur naturally in foods. Such compounds are considered antinutritive factors in foods because of their resistance to digestive enzymes and also because they may be toxic. Thus, catalase may act as a detoxificant which transforms phenolic complexes to nontoxic derivatives (22) as occurs, for example, in the production of instant coffee (4).

In this study, we characterized the catalase activity of *Lactobacillus plantarum* CNRZ 1228, which had been isolated from Egyptian cheese. We used methods including cloning, sequencing, and heterologous expression. We also screened selected LAB strains for the occurrence of the catalase gene and studied the influence of heme, pH, and NaCl levels on the production of catalase in vitro.

The bacterial strains and plasmids used in this study are listed in Table 1. All strains were grown in MRS broth (Merck) at 37°C, except for *Escherichia coli* which was grown in Luria-Bertani (LB) broth (Becton Dickinson) on a rotatory shaker at 200 rpm at 37°C. Solid media were prepared by adding 1.5% agar to the broth media. The strains harboring plasmids with resistance to ampicillin or erythromycin were propagated in media containing 150 μg of ampicillin per ml. The strains harboring plasmids with resistance to erythromycin were propagated in media containing 200 μg of erythromycin per ml for *E. coli* and 50 μg of erythromycin per ml for *Enterococcus faecalis* ATCC 19433.

L. plantarum CNRZ 1228 and various *Lactobacillus* and *Enterococcus* strains isolated from food (Table 2) were screened for catalase activity on MRS agar supplemented with 30 mM hematin (Sigma) by the method of Knauf et al. (9). Catalase activity was detected by flooding the colonies with 3% hydrogen peroxide. *L. plantarum* CNRZ 1228 grown on MRS agar medium did not exhibit any catalase activity unless the medium was supplemented with 30 mM hematin. This result indicated the presence of a heme-dependent catalase activity in *L. plantarum* CNRZ 1228. Total genomic DNA isolation from *L. plantarum* CNRZ 1228 and other LAB was performed by the method of Quadri et al. (14). Large- and small-scale plasmid DNA preparations from *E. coli* (16) and *E. faecalis* (6) were performed by established techniques.

For cloning of the catalase gene, total genomic DNA was partially digested with *Sau*3AI, and the DNA fragments of 1.5 to 2.5, 2.5 to 3.5, 3.5 to 5, and 5 to 7 kb were purified from the agarose gel using the QIAEX II kit (Qiagen). DNA fragments ligated with T4 DNA ligase (New England Biolabs) into the *Bam*HI-cleaved plasmid pBluescript SK+/− (pSKII+/−) were used to transform electrocompetent *E. coli* Top10 cells (Invitrogen) by methods described by Sambrook et al. (16). The gene library constructed this way consisted of about 3,500 clones. The transformants were screened for catalase activity on brain heart infusion (BHI) containing tannic acid (BHI-tannic acid medium) and ampicillin (150 μg/ml), which is the

* Corresponding author. Mailing address: Federal Research Centre for Nutrition, Institute of Biotechnology and Molecular Biology, Haid-und-Neu-Strasse 9, D-76131 Karlsruhe, Germany. Phone: 49 721 6625 225. Fax: 49 721 6625 453. E-mail: Charles.Franz@bfe.uni-karlsruhe.de.

† Present address: Area de Microbiología, Departamento de Ciencias de la Salud, Facultad de Ciencias, Universidad de Jaén, 23071-Jaén, Spain.

TABLE 1. Bacteria and plasmids used in this study

Bacterial strain or plasmid	Description[a]	Source or reference
Bacterial strains		
L. plantarum CNRZ 1228	Catalase positive, tannase positive	This study; 17
E. coli Top10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15Δ*lacX74 deoR recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL*(Str^r) *endA1 nupG*	Invitrogen
E. faecalis ATCC 19433	Plasmidless	
Plasmids		
pSKII+/−	Expression vector, Ap^r, 2.96 kb	New England Biolabs
pUC19	Expression vector, Ap^r, 2.686 kb	Gibco BRL
pMG36e	*E. coli-Lactococcus* shuttle vector, Em^r, 3.6 kb	19
pHA01	pSKII+/− containing 1.4-kb *Bam*HI insert *of L. plantarum* CNRZ 1228 genomic fragment	This work
pHA02	pUC19 containing 1,522-bp *Xba*I-*Sph*I PCR product insert of catalase gene	This work
pHA03	pMG36e containing 1,522-bp insert of *Xba*I-*Sph*I catalase gene	This work

[a] Ap^r, encoding ampicillin resistance; Em^r, encoding erythromycin resistance.

same medium used for screening for tannase activity (12). This medium was used as a plate-screening assay which could detect catalase activity when cloning into catalase-positive bacteria such as *E. coli*. First, the plates were incubated anaerobically for 2 days to allow the colonies to grow and to prevent darkening of the medium; the plates were then kept aerobically for a further 24 h at 37°C to induce the catalase gene (7). A zone of clearing surrounding the colonies, which developed during aerobic incubation, indicated tannic acid degradation as a result of catalase activity. Plasmid DNA (pHA01) of one clone was isolated using the Qiagen Midiprep plasmid purification kit (Qiagen), and the insert was sequenced bidirectionally at GATC Biotech (Konstanz, Germany) using M13 universal oligonucleotide primers and custom primers. Plasmid DNA (pHA01) exhibiting presumptive catalase activity on BHI-tannic acid medium was shown to harbor a 2.5-kb DNA fragment insert.

Analysis of the nucleotide sequence of the 2.5-kb fragment in pHA01 revealed one open reading frame (*katL*) whose sequence comprises 1,455 bp and which encodes a protein of 484 amino acids with a calculated molecular mass of 55 293.93 Da and an isoelectric point of 5.28. A probable ribosome binding site for the catalase gene (GAAAAGGG) was located 8 bases upstream of the initiation codon. Possible −10 (TTT AAA) and −35 (TTGAAG) promoter sequences separated by 22 nucleotides were detected upstream of the structural gene (*katL*). A possible terminator consisting of the inverted repeat sequences TAAAGAGCGACA (right) and TGTCGCTC TTTA (left), capable of forming a stem-loop structure, were located 76 bases downstream of the TAA stop codon. These inverted repeats were separated by 29 bases (data not shown). The amino acid sequence of catalase from *L. plantarum* CNRZ 1228 was almost identical (483 of 484 identical amino acids, i.e., 99.8% identity) to the catalase produced by *L. plantarum* WCFS1 (the complete genome of this strain was recently sequenced [GenBank accession no. AL935262]). Thus, while KatL contained Leu^{396}, the catalase from *L. plantarum* WCFS1 contains a phenylalanine residue at this position. KatL also showed high homology (68% identity, 85.5% similarity) to the catalase of *Lactobacillus sakei* LTH677 (9), especially at the N-terminal region of these proteins.

The *katL* gene was amplified by PCR using primers HA-1 (5′-TAT ATC TAG ACA AGA GCT TTC TTG TAA TTT-3′) and HA-2 (5′-TAT AGC ATG CCG CGC TGG ATA ACA TTC GG-3′) upstream and downstream of this gene and which contained *Xba*I and *Sph*I restriction enzyme sites, respectively (underlined). DNA was amplified in a 50-μl volume containing 100 ng of template DNA, 200 μM concentration of each deoxynucleoside triphosphate, 25 pM concentration of the respective primer, 1.5 U of *Taq* DNA polymerase (Amersham Pharmacia), and 1× *Taq* polymerase buffer (Amersham Pharmacia). DNA was amplified in 32 cycles (denaturation at 94°C for 1 min, annealing at 51°C for 1 min, and extension at 72°C for 2 min), purified, and cut with *Xba*I or *Sph*I. The PCR product was cloned into plasmid pUC19, resulting in plasmid pHA02 which was used to electrotransform *E. coli* Top10 cells. The plasmid DNA from positive clones was used to sequence the catalase gene to confirm the fidelity of the PCR. This nucleotide sequence analysis revealed one error in one base pair, which resulted in the amino acid at position 33 being modified in the PCR-amplified catalase gene (Kat33-His/Ser). However, this mutation resulting from the PCR amplification did not abolish catalytic activity.

For heterologous expression, the PCR-amplified *katL* gene was excised from pHA02 with *Xba*I and *Sph*I and cloned into the *E. coli-Lactococcus* shuttle vector pMG36e, resulting in plasmid pHA03. Plasmid pHA03 was used to transform *E. coli* MH1 (2), and plasmid DNA was isolated from transformants containing pHA03. The plasmid was then transformed by electroporation into the heterologous host *E. faecalis* ATCC 19433. The *katL* gene was successfully expressed in the heterologous host *E. faecalis* ATCC 19433, as it exhibited catalase activity on an MRS plate in the presence of 30 mM hematin. In contrast, the negative-control *E. faecalis* ATCC 19433 containing pMG36e without an insert did not exhibit any catalase activity in MRS agar medium containing 30 mM hematin. This result showed that *katL* was necessary for production of a functional catalase.

TABLE 2. Heme-dependent catalase activity and presence of the catalase gene detected in *Enterococcus* and *Lactobacillus* strains

Strain[a]	Catalase activity	Catalase gene
Enterococcus strains		
E. durans FAIR-E 1, 251, and 364	+	−
E. durans FAIR-E 140	−	−
E. faecium FAIR-E 3, 14, 24, 25, 50, 80, 83, 154, 171, 196, 198, 202, 210, 212, 227, 280, and 349	+	−
E. faecium FAIR-E 6, 26, 34, 160t1, 207, 284, 338, and 362	−	−
E. faecalis FAIR-E 176, 224, 229, 260, 278, 279, 292, 302, 307, 313, 337, 339, and 404	+	−
E. faecalis FAIR-E 35, 77, 265, 325, and 363	−	−
Lactobacillus strains		
L. plantarum ATCC 8014, DSM 20246, and 20174; DC9, DC10, DC254, DC259, DC260, DC261, DC262, and DC1505	+	+
L. sakei DSM 20017 and DC982	+	+
L. sakei Lb706	+	−
L. sakei DC997 and DC1204	−	−
L. pentosus DSM 20314, DC649, and DC797	+	+
L. pentosus CNRZ 1561	+	−
L. curvatus DC1693	+	+
L. curvatus DC432 and DC1205	−	−
L. rhamnosus DC13 and DC53	−	−
L. paracasei subsp. *paracasei* DC8	−	−
L. casei subsp. *casei* DC287	+	+
L. casei subsp. *casei* DC1421 and DC1433	−	−
L. reuteri DSM 20016, DC951, and DC952	+	−
L. reuteri DC882	−	−
L. alimentarius DC878 and DC1168	−	−
L. brevis DC208	+	+
L. buchneri DC398	+	+
L. buchneri DC671	−	−
Weissella confusa DC1733	−	−

[a] Strains were from the following culture collections: FAIR-E, enterococcus culture collection of the FAIR-CT97-3078 European Union Study (18) kept at the BCCM/LMG Bacteria Collection, University of Ghent; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen; ATCC, American Type Culture Collection; CNRZ, CNRZ collection of the Unité de Recherches Laitières et Génétique Appliquée, Institut National de la Recherche Scientifique; DC, Danisco Cultures.

To test whether other LAB isolated from food possess the catalase gene, we first screened these strains (Table 2) for heme-dependent catalase activity on MRS medium containing 30 mM hematin. We found that 75% of *Enterococcus durans* (3 of 4 strains), 64% *Enterococcus faecium* (16 of 25 strains), 72% *E. faecalis* (13 of 18 strains), and 66% of lactobacilli strains (25 of 38 strains) investigated in this study exhibited catalase activity when exogenous heme was supplied. Total genomic DNA isolated from various positive strains was used as a template for amplification of the catalase gene. For PCR amplification, the primers Cat-1 (5′-CAA AAT ATG CAA TGG GAT TT-3′) and Cat-2 (5′-ACC TTG TAG TAA TTT GTC GGG-3′) were used. PCR amplification was done as mentioned above in 35 cycles (denaturation at 94°C for 1 min, annealing at 48°C for 1 min, and extension at 72°C for 40 s). Genomic DNA from *L. plantarum* CNRZ 1228 was used as a positive control. PCR amplicons of the catalase gene could be obtained only when DNA from *L. plantarum* strains DC9, DC10, DC254, DC259, DC260, DC261, DC262, DC1505, ATCC 8014, DSM 20246, and 20174, *Lactobacillus pentosus* strains DSM 20314, DC649, and DC797, *L. sakei* strains DSM 20017 and DC982, and *Lactobacillus curvatus* DC1693, *Lactobacillus casei* subsp. *casei* DC287, *Lactobacillus brevis* DC208, and *Lactobacillus buchneri* DC398 were used as templates in PCRs. For all other strains, including enterococci and other lactobacilli, no PCR product could be amplified (Table 2). Therefore, the specific primers used in this study were speculated to anneal to regions which are conserved only in the catalase genes of some lactobacilli. Consequently, the heme-dependent catalase genes in enterococci may be expected to show little homology in the gene regions to which the primers were targeted, and these catalase genes may be expected to be heterogenous in primary sequence from those produced by some lactobacilli.

For a possible application of LAB as starter cultures in sausage fermentation, knowledge about their potential to form hydrogen peroxide and their production of active catalase under various ecological conditions may be required. The influence of pH and heme and NaCl concentrations on catalase activity in *L. plantarum* CNRZ 1228 was determined in vitro in triplicate. Catalase activity was detected by the method of Rorth and Jensen (15), using a WTW oxygraph (OXI DIGI 550) equipped with a Clark oxygen electrode (Trioxmatic EO 166). The assays were all done aerobically in a stirred 250-ml flask containing 200 ml of H_2O_2 buffer (22 mM H_2O_2 in 0.15 M phosphate buffer [pH 7.0]) and by measuring the O_2 released by 10^8 CFU of bacteria/ml (10-ml culture of *L. plantarum* CNRZ 1228 grown overnight in MRS broth under different environmental conditions). *L. plantarum* CNRZ 1228 grown aerobically in MRS broth exhibited catalase activity at all hematin concentrations tested, but the highest activity of 203 ± 0.5 μM O_2/liter/min was detected in the range of 20 to 50 ppm (31 to 79 μM) of hematin added to the growth medium. Accordingly, 20 ppm of hematin was chosen in the further tests.

The aerobic growth of *L. plantarum* CNRZ 1228 at 37°C for 24 h caused a decrease in the pH of MRS broth to pH 3.8 as a result of acid production. However, this final pH did not affect the catalase activity (203 ± 0.3 μM O_2/liter/min with 20 ppm of hematin [result not shown]). However, the effect of the initial pH of the MRS growth medium was presumed to influence catalase activity, so it was investigated. The initial pH indeed influenced the catalase activity of *L. plantarum* CNRZ 1228. At a pH range of 4.9 to 5.7 after sterilization of the medium, catalase activity was detected, reaching a maximum at pH 5.4 (245.7 ± 0.6 μM O_2/liter/min), followed by a slight decrease in activity at pH 5.7 (203 ± 0.3 μM O_2/liter/min). The lowest activity was at pH 4.9 (147.7 ± 0.4 μM O_2/liter/min). However, in MRS broth supplemented by hematin at the initial pH values of 4.0 and 4.5, no growth and hence no catalase activity was detected in cultures incubated at 37°C for up to 48 h. In the pH range from 4.9 to 5.7, the growth yield (5×10^8 CFU/ml after 24 h of incubation at 37°C) was not affected by the pH of the culture medium (data not shown).

The catalase activity of *L. plantarum* CNRZ 1228 was also

dependent on the concentration of salt. The presence of increasing NaCl concentrations (0 to 5%) and 20 ppm of hematin in the growth medium did not affect the growth yield (4×10^8 CFU/ml after 24-h incubation at 37°C). Catalase activity was constant (203 ± 0.7 μM O_2/liter/min) with 0 to 4% NaCl but decreased at 5% NaCl (51.81 ± 0.03 μM O_2/liter/min). At 6 to 7% NaCl, no growth and hence no catalase activity could be detected after 24 h of incubation at 37°C, while after 48 h, both growth (10^8 CFU/ml) and low catalase activity (20 μM O_2/liter/min) were detected. These results may also imply that the lack of growth at pH 4.5 and 6% salt result from the presence of hematin in the medium. Sausage fermentations start at a pH of 5.8 and reach a final pH of about 4.9 to 5.0. Therefore, the catalase activity would not be impaired by pH conditions encountered in sausage fermentations, as our results indicate that catalase production by *L. plantarum* CNRZ 1228 occurred in the range of pH 4.9 to 5.7. The salt content in standard fermented sausage production is about 3 to 4.5%, indicating that for fermentation of sausages, catalase activity would not be inhibited by salt.

The *L. plantarum* CNRZ 1228 culture and the pure catalase from Sigma were tested for their ability to degrade phenolic compounds that naturally occur in foods. BHI agar plates (Becton Dickinson) supplemented with 0.5% yeast extract (Merck) and overlaid with 5 ml of filter-sterilized 2% tannic acid (ICN Biomedicals) were used for tannic acid degradation tests by the method of Osawa (12). Other phenolic substrates tested individually in plate assays included gallic acid (10 g/liter) (Sigma), gallic acid methyl ester (10 g/liter) (ICN Biomedicals), coffein (10 g/liter) (Sigma), and guaiacol (0.2 g/liter) (Sigma) added to the MRS agar medium before sterilization. Samples (5 μl) of a culture of *L. plantarum* CNRZ 1228 grown overnight in MRS broth at 37°C were spotted onto the surfaces of plates containing the different phenolic compounds at the concentrations described above, and the plates were incubated aerobically at 30°C for 3 days. For a control, pure catalase (Sigma) from bovine liver (210 μg/ml) was used. This concentration was determined as the minimum to obtain activity in the agar spot assays. Degradation of the phenolic compounds was indicated by a clear zone surrounding the colony growth. The catalase produced by the *L. plantarum* CNRZ 1228 culture during growth and the pure catalase were shown to degrade gallic acid methyl ester, gallic acid, and also tannic acid bound to proteins as determined by zones of clearing in plate assays. However, both coffein and guiaicol were less accessible to the degradative effect of catalase, as no zones of clearing were detected in plate assays.

Nucleotide sequence accession number. The nucleotide sequence of the chromosomal DNA fragment from *L. plantarum* CNRZ 1228 containing the catalase gene was submitted to GenBank and was given the accession number AY375759.

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