

Myosin-cross-reactive antigens from four different lactic acid bacteria are fatty acid hydratases

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Abstract The 67 kDa myosin-cross-reactive antigen (MCRA) is a member of the MCRA family of proteins present in a wide range of bacteria and was predicted to have fatty acid isomerase function. We have now characterised the catalytic activity of MCRA from four LAB stains, including *Lactobacillus rhamnosus* LGG, *L. plantarum* ST-III, *L. acidophilus* NCFM and

Bifidobacterium animalis subsp. *lactis* BB-12. MCRA genes from these strains were cloned and expressed in *Escherichia coli*, and the recombinant protein function was analysed with lipid profiles by GC–MS. The four MCRA catalysed the conversion of linoleic acid and oleic acid to their respective 10-hydroxy derivatives, which suggests that MCRA proteins catalyse the first step in conjugated linoleic acid production. This is the first report of MCRA from *L. rhamnosus* with such catalytic function.

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Introduction

The 67 kDa myosin-cross-reactive antigen (MCRA) was first identified in *Streptococcus pyogenes* and was predicted to have polyunsaturated fatty acid (PUFA) isomerase function (Kil et al. 1994). MCRA comprise a family of proteins that are present in a wide range of bacteria, especially lactic acid bacteria. They show high homology with linoleate isomerase from *Lactobacillus reuteri* PYR8 (Rosson et al. 2004), which transforms linoleic acid into conjugated linoleic acid (CLA). MCRA have fatty acid hydratase activity and that the MCRA from *S. pyogenes* (Volkov et al. 2010) and *Bifidobacterium breve* (Rosberg-Cody et al. 2011) are FAD-containing enzymes that act as hydratases on

C₉ bonds of C-18 free fatty acids, producing 10-hydroxy derivatives. Kishino et al. (2011) reported that MCRA from *L. plantarum* AKU 1009a is present in the cell membrane fraction and that it transforms linoleic acid into 10-hydroxy-*cis*-12-octadecenoic acid (10-HOE).

The mechanism for the microbial production of CLA using *L. acidophilus* has been discussed and involves hydroxyl fatty acids as intermediates. The MCRA from *L. plantarum* (Kishino et al. 2011) is, with two other enzymes, a multi-component enzyme system for producing CLA, and CLA formation consists of three distinct steps: hydration of linoleic acid to 10-HOE, then isomerisation and dehydration of 10-HOE into CLA.

Previously, we confirmed the CLA production capacity of four bacterial strains (*L. rhamnosus*, *L. plantarum*, *L. acidophilus*, and *B. animalis* subsp. *lactis*), and all of them did indeed produce CLA with 10-HOE accumulation. The aim of present study was to investigate the MCRA proteins from these four lactic acid bacteria.

Materials and methods

Materials

Linoleic acid and oleic acid were purchased from Sigma–Aldrich. All other chemicals used were of analytical grade and are commercially available.

Microorganism cultivation and preparation of washed cells

Lactobacillus rhamnosus LGG, *L. plantarum* ST-III and *L. acidophilus* NCFM were aerobically cultured three times in de Man, Rogosa and Sharpe (MRS) culture medium consisting of 1 % Tryptone, 1 % meat extract, 0.5 % yeast extract, 2 % glucose, 0.1 % Tween 80, 0.2 % K₂HPO₄, 0.5 % sodium acetate, 0.2 % diammonium citrate, 0.02 % MgSO₄·7H₂O and 0.005 % MnSO₄·H₂O (pH 6.5) at 37 °C for 24 h. *B. animalis* subsp. *lactis* BB-12 was cultured in MRS supplemented with 0.05 % (w/v) L-cysteine-HCl. *E. coli* BL21 (DE3) carrying the plasmid pET28a was routinely cultured aerobically in Luria-Bertani (LB) medium (10 g Tryptone/l, 5 g yeast extract/l, 10 g NaCl/l) at 37 °C in the presence of kanamycin (50 µg/ml) as a selective marker.

DNA manipulations and plasmid construction

Genomic DNAs were isolated from the four strains as described by Hoffman and Winston (1987) with some modifications, adding lysozyme into the breaking system and treating the aqueous layer with protease K for 30 min after breaking. MCRA genes from *B. animalis* subsp. *lactis* BB-12 (GenBank: ADC85468), *L. rhamnosus* LGG (GenBank: YP_003170249.1), *L. plantarum* ST-III (GenBank: YP_003923433) and *L. acidophilus* NCFM (GenBank: AAV42528.1) were amplified from genomic DNAs with specific primers. PCR was performed with KOD-plus DNA polymerase (Toyobo, Japan) according to the manufacturer's protocol. PCR conditions were as follows: 30 cycles of 45 s denaturation (94 °C), 30 s annealing (60 °C), and 2.5 min elongation (68 °C). For expression in *E. coli*, the MCRA ORF was amplified with the primers listed in Supplementary Table 1 and cloned into the pET28a expression vector (Novagen, USA), yielding plasmids pET28a-LGG, pET28a-ST-III, pET28a-NCFM and pET28a-BB12 (N-His-tagged version).

Production and purification of recombinant MCRA proteins

For protein production, *E. coli* BL21 Star strain (Invitrogen) separately harbouring pET28a-LGG, pET28a-ST-III, pET28a-NCFM and pET28a-BB12 plasmids were used. Bacteria were cultivated in LB medium supplied with kanamycin (50 µg/ml) at 37 °C until OD₆₀₀ reached 0.6. At that point, IPTG was added at 0.1 mM, and the culture was placed at 20 °C for 20-h induction of protein expression.

For protein purification, cells were then harvested by centrifugation, washed with buffer A (0.1 M Tris/HCl, pH 6.5, 0.1 M NaCl), resuspended in the same buffer containing 1 mg lysozyme/ml, 1 mM PMSF, 1 µg DNase/ml and 10 mM MgCl₂, and ultra-sonicated. The cell debris was removed by centrifugation. The His₆-tagged fusion proteins were purified by nickel ion affinity chromatography with a chelating Sepharose Fast Flow column. Fusion proteins were eluted with 5 ml elution buffer (0.1 M Tris/HCl, pH 6.5, 0.1 M NaCl and 500 mM imidazole) and dialysed overnight against 0.1 M Tris/HCl buffer containing 20 % (v/v) glycerol (pH 6.5) at 4 °C. The protein concentration was determined from the supernatant by the Bradford method. Purified proteins were stored at –80 °C.

Immunoblotting

Protein, 10 µg, was subjected to SDS–PAGE (12 % gel) followed by protein transfer to a PVDF membrane; the immunoblots were developed with the use of anti-His antibody at a dilution of 1:1,000 (Tiangen, Beijing, China). Horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Tiangen) secondary antibody diluted at 1:500 was used for the detection of specific antibody binding. The bands were visualised with enhanced chemiluminescence reagents (Kangwei, Beijing, China) according to the manufacturer’s instructions.

Activity assay and fatty acid analysis

To demonstrate the enzymatic activity of recombinant MCRA protein, 50 µg linoleic acid or oleic acid was mixed with 10 µg purified protein in 1 ml buffer A and incubated for 1 h at 25 °C. Lipid extraction and preparation of fatty acid methyl esters (FAMES) were performed according to the procedures of Bligh and

Dyer (1959). Nonadecanoic acid was used as the internal standard. Free fatty acids were dissolved in methanol and converted to corresponding methyl esters with (trimethylsilyl)-diazomethane. FAMES were extracted with n-hexane and analysed by GC–MS on a Finnigan Trace gas chromatograph (Thermo, USA) equipped with a Finnigan Trace mass spectrometer (Thermo). Injections of 10 µl were performed automatically at a split ratio of 62:1. Hydrogen and helium were used as the carrier gases through an Agilent DB-WAX column (30 × 0.25 mm, i.d. 0.25 µm; Agilent, USA). The column temperature was set initially at 180 °C for 0.5 min, then increased to 230 °C in increments of 5 °C/min. The 230 °C was maintained for 13 min. The injector and detector were operated at 240 °C. Electron energy of 70 eV and ion source temperature of 230 °C were used.

Bioinformatics analysis

To analyse the four MCRA proteins, PSORTb, Conserved Domain Search, Pfam Search and Motif Scan

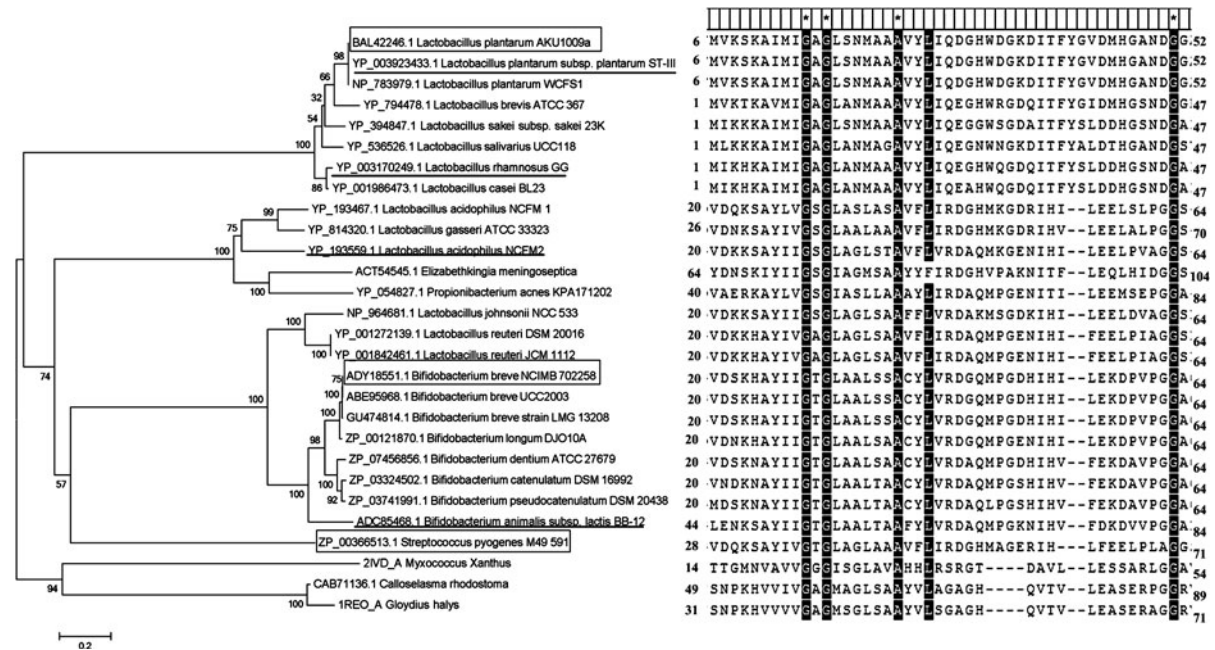


Fig. 1 Phylogenetic tree and multiple sequence alignment of FAD-binding domain of MCRA and oxidoreductases. On the left, the evolutionary relationships of MCRA proteins are shown. The scale bar represents an evolutionary distance of 0.2 amino acid substitutions per site. The four MCRA proteins (BB12, LGG, NCFM, ST-III) are underlined. Three MCRA proteins, reported to have fatty acid hydratase function, are indicated with a box (*B.*

breve NCIMB 702258, *L. plantarum* AKU 1009a, *S. pyogenes* M49). On the right, alignment of the FAD-binding motif from these corresponding proteins is shown. Residues that are identical in all the sequences are highlighted. Positions of the start and the end of the protein region are shown for every sequence on either the left or right side of the alignment

were used. Homologs to the previously identified fatty acid hydratase from *S. pyogenes* M49 GenBank: ZP_00366513.1) and *B. breve* NCIMB 702258 (GenBank: ADY18551.1) were selected from BLAST analysis (Altschul et al. 1990). ClustalW2 (Larkin et al. 2007) was used for multiple sequence alignment, and protein phylogenetic trees were constructed with MEGA5 (Tamura et al. 2011).

Results and discussion

Sequence analysis and phylogenetic analysis

All four proteins were located in the cytoplasm. The calculated molecular weights were 64,444.08 Da (LGG), 64,751.24 Da (ST-III), 67,617.50 Da (NCFM) and 82,421.61 Da (BB12). Comparison with amino acid sequences in the database revealed that the four MCRA proteins showed more than 30 % homology with the MCRA from *S. pyogenes* M49 (LGG: 33 %, ST-III: 32 %, NCFM: 68 % and BB12: 47 %), and at least 29 % homology with the MCRA from *B. breve* NCIMB 702258 (LGG: 29 %, ST-III: 30 %, NCFM: 51 %, and BB12: 68 %). Figure 1 displays a phylogenetic analysis of the four MCRA proteins along with oleate hydratase homologs. The evolutionary relationship further supports the MCRA as fatty acid hydratases.

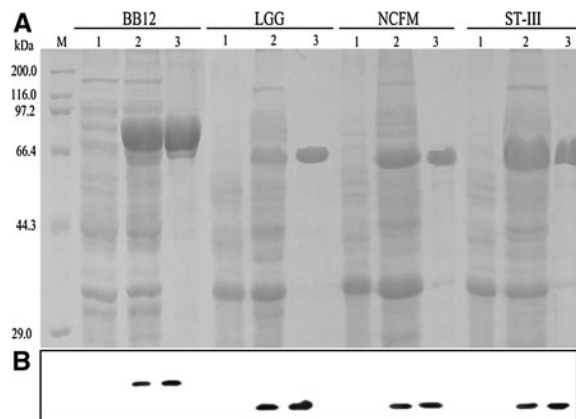


Fig. 2 Results of SDS-PAGE and Western blot analyses of the recombinant proteins (BB12, LGG, NCFM and ST-III). **a** SDS-PAGE of BB12, LGG, NCFM and ST-III. **b** Western blotting of the control, crude extracts and purified proteins. Lane: M: Marker; 1: Non-induced control; 2: Crude extracts; 3: Purified protein

The majority of amino acid residues that interact with FAD are conserved (Fig. 1). The detailed multiple sequence alignment of MCRA presents a GxG(x)₇-L(x)₁₇₋₂₃G motif consistent with FAD- and NAD(P)-binding Rossmann folds (Kleiger and Eisenberg 2002). The combined results of Pfam Search, Conserved Domain Search and Motif Scan showed that the four proteins contained a Strep_67 kDa_ant domain, which was the original domain of the MCRA family and an FAD-binding motif. Typically, the MCRA of *B. animalis* subsp. *lactis* BB-12 and *L. acidophilus* NCFM have an extra FAD-dependent oxidoreductase domain, which is also found in the linoleate isomerase from *Propionibacterium acnes* (Liavonchanka et al. 2006) and might remove electrons from the PUFA substrate and transfer them to O₂ or redox cofactors.

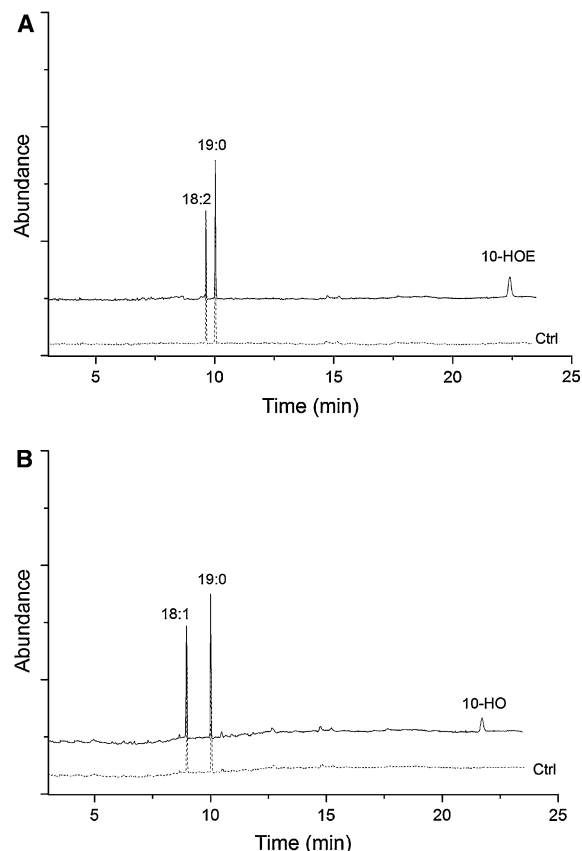


Fig. 3 GC-MS total ion chromatograms of the products from linoleic acid and oleic acid upon incubation with the recombinant MCRA proteins. **a** Products formed from linoleic acid and its control. **b** Products formed from oleic acid and its control. Control: linoleic acid or oleic acid incubated in buffer A without protein extract for 1 h at 25 °C

According to previous reports (Volkov et al. 2010; Rosberg-Cody et al. 2011), MCRA proteins from *S. pyogenes* and *B. breve* are FAD-dependent; FAD is not involved in the catalysing reaction but plays a role in protein stability. There are other flavoenzymes that require FAD to maintain their proper protein architecture but without FAD being directly involved in the enzymatic reaction (Cromartie and Walsh 1976; Vargo et al. 1981; Liavonchanka et al. 2006). FAD in the four MCRA proteins might play the same role as previously reported.

Expression and purification of the recombinant MCRA proteins

The four MCRA proteins were overproduced in pET28a in *E. coli* and purified on the Ni²⁺-NTA column (Fig. 2a). Major bands with apparent molecular weights of 64 kDa (LGG), 64 kDa (ST-III), 67 kDa (NCFM) and 82 kDa (BB12) were visualised by SDS-PAGE after elution, and these corresponded

to the expected molecular weights of the four proteins, which were absent before IPTG induction. Immunoblotting with anti-His-tag antibodies confirmed that each single band contained a 6×His tag (Fig. 2b).

Production of recombinant proteins in *E. coli* and enzymatic assays

The four recombinant proteins converted linoleic acid and oleic acid into 10-HOE and 10-hydroxy-octadecanoic acid (10-HO), respectively (Fig. 3a, b). Both products showed two significant MS fragments at 169 m/z and 201 m/z, but there is a difference at 143 m/z (Fig. 4a, b). Combined with the fragmentation pattern (Fig. 4c, d), the 143 m/z peak distinguished the two products; 10-HOE lacked this significant peak. Two different oleic acids, *cis*-9-oleic acid and *trans*-9-oleic acid, were used as substrates, but the latter could not be converted (data not shown). All the results were in line with that of *B. breve* NCIMB 702258 (Rosberg-Cody et al. 2011), which

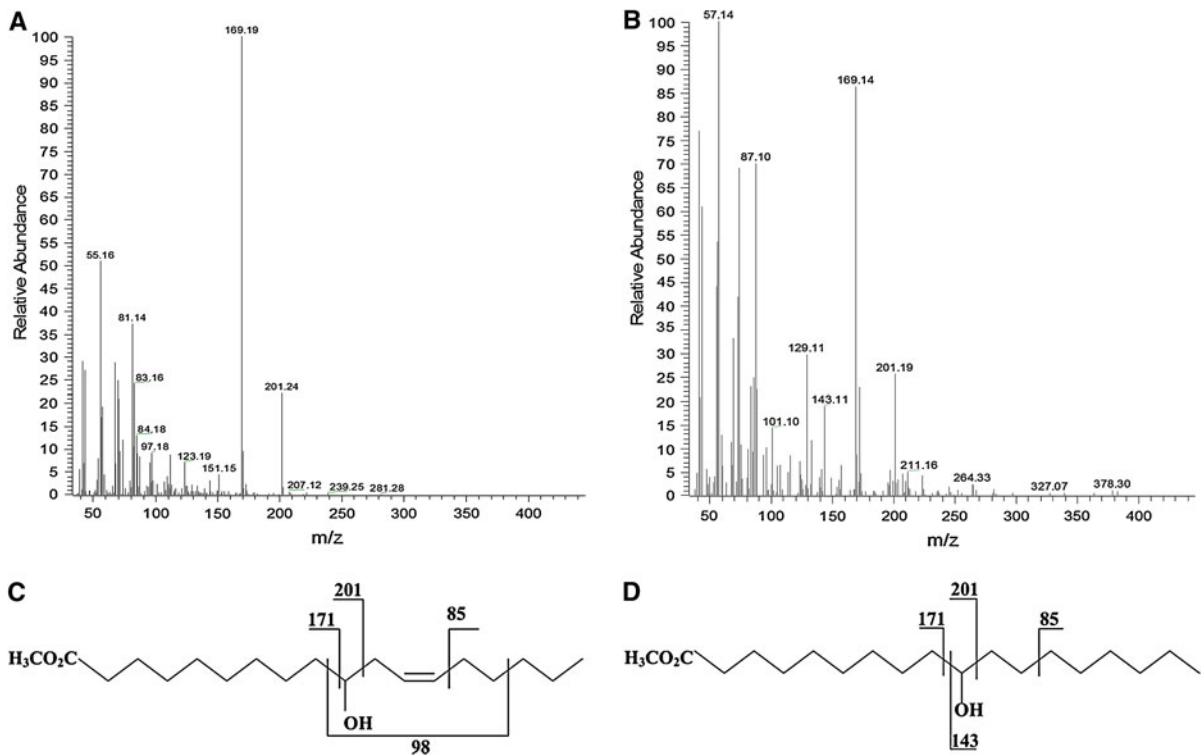


Fig. 4 MS fraction of the products of the reaction with recombinant MCRA protein with free linoleic acid and oleic acid. **a** Mass spectra of 10-HOE obtained after incubation of

linoleic acid. **b** Mass spectra of 10-HO obtained after incubation of oleic acid. **c** The structure of 10-HOE and its fragmentation pattern. **d** The structure of 10-HO and its fragmentation pattern

can catalyse 10-HOE formation from linoleic acid and 10-HO from cis-9-oleic acid, respectively. Besides, no further products were transformed from hydroxyl derivatives by MCRA in our results was consistent with that of *S. pyogenes* (Volkov et al. 2010).

The first attempt to identify the enzymatic activity of an MCRA from *L. reuteri* PYR8 was reported by Rosson et al. (2004) whose aim was to confirm its linoleic acid isomerase function, but no isomerase activity was detected. MCRA from *S. pyogenes* was heterologously expressed in *E. coli* and there was no CLA-forming isomerase activity (Feussner et al. 2008). We did not detect the linoleate isomerase activity from the MCRA proteins either but could confirm production of CLA in the four lactic acid bacteria; indeed, the four strains produced CLA with high concentrations of 10-HOE, although the accumulation of CLA was not at the same level in each of the four strains.

Our results were similar to a previous report (Ogawa et al. 2001) that the washed *L. acidophilus* AKU 1,137 cells accumulated CLA from linoleic acid via 10-HOE as an intermediate. According to Kishino et al. (2011), the MCRA protein from *L. plantarum* AKU 1009a converted linoleic acid to 10-HOE, and the product was finally transformed into CLA with two other enzymes. Our current results further illustrate the mechanism of CLA production in lactic acid bacteria, both *Lactobacillus* and *Bifidobacterium*, and suggest that the four lactic acid bacteria produce CLA with a multi-component enzyme system, similar to that of *L. plantarum* AKU 1009a. The remaining enzymes will be further investigated.

Conclusions

MCRAs from *B. animalis* subsp. *lactis*, *L. rhamnosus*, *L. plantarum* and *L. acidophilus* were cloned, expressed, and purified. Each protein can be classified as a fatty acid hydratases, converting linoleic acid and oleic acid to 10-HOE and 10-HO, which represents the first step in CLA production. This is the first report of the CLA production capacity of *L. rhamnosus* LGG and the MCRA function from *L. rhamnosus*.

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