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Identification and characterization of an oleate hydratase-encoding gene from *Bifidobacterium breve*

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Abbreviations: MCRA, myosin cross reactive antigen; OA, oleic acid; LA, linoleic acid; 10-HSA, 10-hydroxystearic acid; LAI, linoleic acid isomerase

Bifidobacteria are common commensals of the mammalian gastrointestinal tract. Previous studies have suggested that a bifidobacterial myosin cross reactive antigen (MCRA) protein plays a role in bacterial stress tolerance, while this protein has also been linked to the biosynthesis of conjugated linoleic acid (CLA) in bifidobacteria. In order to increase our understanding on the role of MCRA in bifidobacteria we created and analyzed an insertion mutant of the MCRA-encoding gene of *B. breve* NCFB 2258. Our results demonstrate that the MCRA protein of *B. breve* NCFB 2258 does not appear to play a role in CLA production, yet is an oleate hydratase, which contributes to bifidobacterial solvent stress protection.

Introduction

Bifidobacteria are Gram positive, non-motile, non-sporulating, anaerobic rods which possess a high G+C genome content and belong to the phylum *Actinobacteria* and the family *Bifidobacteriaceae*.[1,2] Bifidobacteria were first isolated more than a century ago[3] and naturally inhabit the gastrointestinal tract of humans and other mammals, being particularly abundant in breast-fed infants.[4,5] Bifidobacteria have been claimed to maintain gastrointestinal health[6] and are therefore used as a health-promoting or probiotic bacterial ingredients in certain functional foods.[7] Their reported beneficial effects on the host include inhibition of pathogens, alleviating lactose intolerance, enhancing natural immunity and reducing serum cholesterol.[8-10]

Bifidobacteria have been shown to convert oleic acid (OA) to 10-hydroxystearic acid (10-HSA).[11] OA is a mono-unsaturated fatty acid that occurs naturally in vegetable oils[12] and is reported to confer a range of health benefits including the inhibition of adrenoleukodystrophy (ALD), a metabolic disorder that leads to demyelination in the central and peripheral nervous system[13] and reduction in blood pressure.[14] Microbial hydration of an unsaturated fatty acid was first reported for *Pseudomonas* sp 3266 (now known as *Elizabethkingia meningoseptica*), which was shown to possess the ability to convert OA to 10-HSA.[15] However, the isolation and biochemical characterization of the enzyme responsible for this conversion, the oleate hydratase, was not reported until relatively recently.[16] The latter authors also showed that expression of the *E. meningoseptica* OA hydratase, was strongly upregulated when this bacterium was grown in the presence of 0.3% OA. Unsaturated fatty acids such as OA have been shown to be toxic to many bacteria,[17] which is probably due to the disruptive effect of these hydrophobic compounds on the bacterial membrane,[18] combined with their inhibitory action on enoyl-ACP reductase (FabI) and thus fatty acid biosynthesis.[19] The hydration of unsaturated fatty acids is therefore hypothesized to represent a detoxification mechanism in bacteria to aid survival in environments rich in unsaturated fatty acids.[20]

The first MCRA protein (MW ~67 kDa) was identified in *Streptococcus pyogenes*, while screening for antigens recognized by acute rheumatic fever sera. Its amino acid sequence did, at that time, not exhibit similarity to any streptococcal protein with a known function, although it was found to be conserved among pathogenic groups A, C and G of Streptococci.[21] The MCRA protein of *Streptococcus pyogenes* M49 was recently shown to represent a flavin adenine dinucleotide (FAD) enzyme which acts as a fatty acid hydratase.[20] This latter enzymatic activity catalyzes the conversion of OA into 10-hydroxystearic acid, yet the role of the FAD-binding residues present in this and other (predicted) fatty acid hydratases/MCRA-like proteins are not fully understood.[12]

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Table 1. Bioconversion of linoleic acid to CLA by bifidobacterial strains

			CLA converted from 0.5mg ml^{-1} linoleic acid*		
Strain	**Species**	**Source**	***c*9,*t*11**	**S.D**	**% Converted**
Bifidobacterium breve	UCC2003	Isolate from a nursling stool	0.008	0.001	1.73
	NCIMB 8807	Isolate from a nursling stool	0.085	0.011	17.16
	NCFB 2257	Isolate from infant intestine	0.027	0.006	5.54
	NCTC 11815	Isolate from infant intestine	0.148	0.011	29.64
	NCFB 2258	Isolate from infant intestine	0.245	0.021	49.00
	NCIMB 8815	Isolate from infant feces	0.077	0.021	15.54
	JCM 7017	Isolate from human feces	0.229	0.027	45.90
	UCC2005	Isolate from human feces	0.179	0.033	35.88
	UCC2007	Isolate from human feces	0.054	0.003	10.84
	Nizo 658	Isolate from a nursling stool	0.083	0.004	16.70
	LMG 13208	Isolate from infant intestine	0.055	0.00	11.16
	UCC1	Isolate from human feces	0.005	0.000	1.05
	NCFB 2258-MCRA	This study	0.213	0.002	42.60
Bifidobacterium longum					
	UCC2	Isolate from human feces	0	0	0
	UCC3	Isolate from human feces	0	0	0
	KJOC1	Isolate from infant feces	0	0	0
	KJOC2	Isolate from infant feces	0	0	0

*Values represent the average of two independent experiment.

MCRA-like proteins show over 50% sequence similarity to the linoleic acid isomerase (LAI) from *Lactobacillus acidophilus* and *Lb. reuteri* PYR8.[22] LAI catalyzes the conversion of linoleic acid (LA, a dienoic unsaturated fatty acid) to *cis*-9 *trans*-11 (*c*9,*t*11) conjugated linoleic acid (CLA). CLA is the collective term used to describe a group of polyunsaturated fatty acids that exist as positional and geometric stereoisomer's of octadecadienoic acid.[23] Twenty-eight different CLA isomers have been identified, of which the most abundant is the *c*9,*t11* isomer, representing approximately 80% of total CLA in food products. CLA is found naturally in ruminant food products, for example lamb, beef and dairy, due to the process of biohydrogenation of LA in the rumen.[24] The LAI protein is a membrane-bound enzyme which was first isolated from *Butyrivibrio fibrisolvens*.[25] Subsequently, the crystal-structure of an LAI from *Propionibacterium acnes* was solved[26] and the reaction mechanism characterized.[27]

It was recently suggested[11] that the MCRA-like protein produced by bifidobacteria is responsible for the first step of two-step CLA production process in which hydroxy-fatty acids act as intermediates in the production of CLA.[28] In the latter study, the gene encoding the MCRA-like protein from *B. breve* NCFB 2258 was cloned, sequenced and expressed in two heterologous hosts (*Lactococcus* and *Corynebacterium*), and the recombinant proteins assessed for enzymatic activity against fatty acid substrates. It was demonstrated that heterologous expression of this bifidobacterial MCRA in *Lactococcus* and *Corynebacterium* resulted in increased amounts of hydroxy-fatty acids in the culture medium. It was also observed that these recombinant hydroxy-fatty acid-producing cells were more resistant to heat and solvent stress as compared with their non-recombinant, wild type controls.

The aim of the current study was to investigate the biological function of the MCRA-like protein in *Bifidobacterium breve*. The obtained results show that the *B. breve* MCRA-like protein functions as an oleate hydratase, which plays a role in stress tolerance in bifidobacteria, though it does not appear to play a role in CLA production in *B. breve*.

Results

Analysis of CLA production by various *Bifidobacterium* species. The ability of various strains of bifidobacteria to convert LA to CLA was analyzed using a rapid spectrophotometer-based method.[29] Of the 17 bifidobacterial strains tested 11 strains were capable of LA to CLA bioconversion. *B. longum* strains KJOC1, KJOC2, UCC2 and UCC3 and *B. breve* strains UCC1 and UCC2003 did not appear to produce appreciable amounts of CLA from free LA.

Due to the fact that the spectrophotometer-based method merely gives an estimation of CLA content, a more accurate gas liquid chromatography (GLC) method[30] was adopted. Our results demonstrate that *B. breve* NCFB 2258 was the strain with the highest CLA (*c*9,*t*11) conversion capability, which was in keeping with the rapid spectrophotometer results in terms of high and low CLA producers (**Table 1**). However, *B. breve* UCC2003 and *B. breve* NCIMB 8807 were shown to exhibit different CLA production capabilities even though *B. breve* UCC2003 is a clonal isolate of *B. breve* NCIMB 8807.[31] Upon

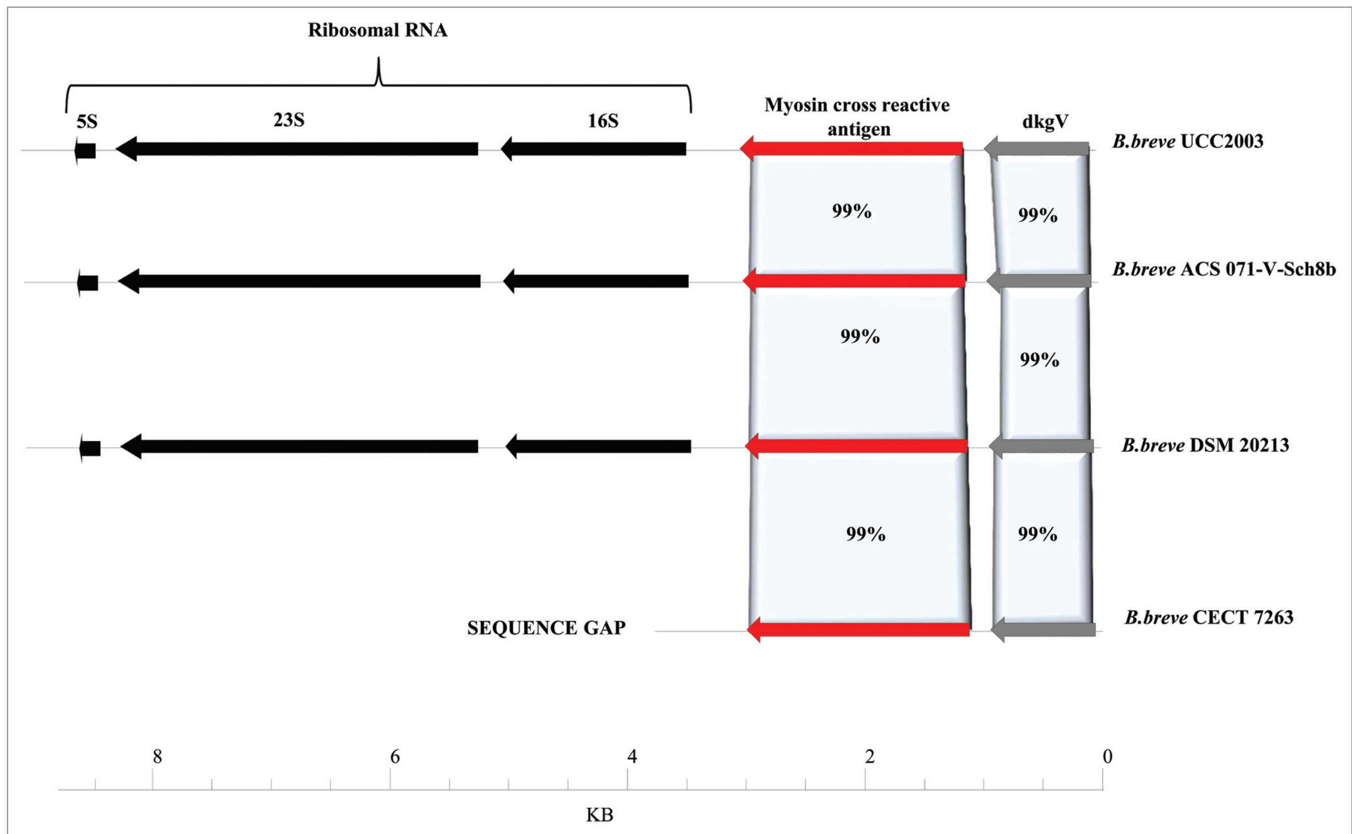

Figure 1. Schematic representation of the comparison of the myosin cross reactive antigen encoding gene, $MCRA_{UCC2003}$, from *B. breve* UCC2003 to other sequenced *B. breve*.[54]

close examination *B. breve* NCIMB 8807 was shown to gradually lose its CLA-producing capability upon repeated sub-cultivation (results not shown). Although the reasons for this unstable CLA-production phenotype are not clear, other CLA-producing strains used in this study did not exhibit such behavior.

Identification and analysis of a *B. breve* UCC2003 myosin cross reactive encoding gene. A gene designated $MCRA_{ucc2003}$, predicted to encode a protein with similarity to other myosin cross reactive antigen (MCRA) proteins was identified from the annotation of the genome sequence of *B. breve* UCC2003.[32] At protein level, the predicted product of $MCRA_{ucc2003}$ is almost identical (99% identity) to predicted MCRAs encoded by homologs on the genomes of *Bifidobacterium breve* CETC 7263 (GenBank accession number ADY18551.1), DSM 20213 (GenBank accession number ZP_12586529.1), ACS-071-V-Sch8b (GenBank accession number ZP_06596910.1), while also displaying high levels of identity (80–99%) to annotated MCRA-encoding genes from a range of other bifidobacteria. An exception to this is found for *MCRA* homologs on the genomes of *B. animalis* subsp *lactis* HN019 (GenBank accession number ZP 02963377) and *B. animalis* subsp *animalis* ATCC 25527 (GenBank accession number YP 006279649.1), where just 68% identity is observed compared with $MCRA_{ucc2003}$. The apparently monocistric $MCRA_{ucc2003}$ (1,878 bp) on the *B. breve* UCC2003 genome corresponds to a deduced protein of 625 amino acids (Mw ~70.5 kDa) and is located upstream of a ribosomal RNA (rRNA) operon and downstream from a gene predicted to encode a 2,5-diketo-D-gluconic acid reductase A (**Fig. 1**).

A mutation in the MCRA-like gene does not affect CLA production in *B. breve* NCFB 2258. As mentioned above, MCRA-like proteins exhibit over 50% sequence similarity to LAI from *Lactobacillus acidophilus* and *Lb. reuteri* PYR8,[22] which have been implicated in the conversion of LA to *c*9,*t*11 CLA. It was recently also suggested[11] that these MCRA-like proteins are responsible for the first step of a proposed two-step CLA production process in which hydroxy-fatty acids act as intermediates in the production of CLA.[28] In order to determine if the MCRA-encoding gene is involved in CLA production, an insertion mutant was created in the MCRA-encoding gene, designated here as $MCRA_{NCFB2258}$, of *B. breve* NCFB 2258 after which the CLA production capability of the mutant strain was analyzed. Based on the rapid spectrophotometer method of[29] the *B. breve* NCFB 2258-MCRA insertion mutant was shown to produce the same amount of CLA as the parent strain *B. breve* NCFB 2258, and this result was verified by GLC analysis (**Table 1**). These data convincingly demonstrate that the MCRA-encoding gene does not play a role in CLA production by *B. breve* NCFB 2258, at least under the conditions tested.

Effect of the MCRA insertion mutation on hydratase activity of *B. breve* NCFB 2258. Bifidobacteria have been shown to exhibit hydratase activity, which allows such bacteria to convert oleic acid into 10-hydroxyoctadecanoic acid otherwise known as

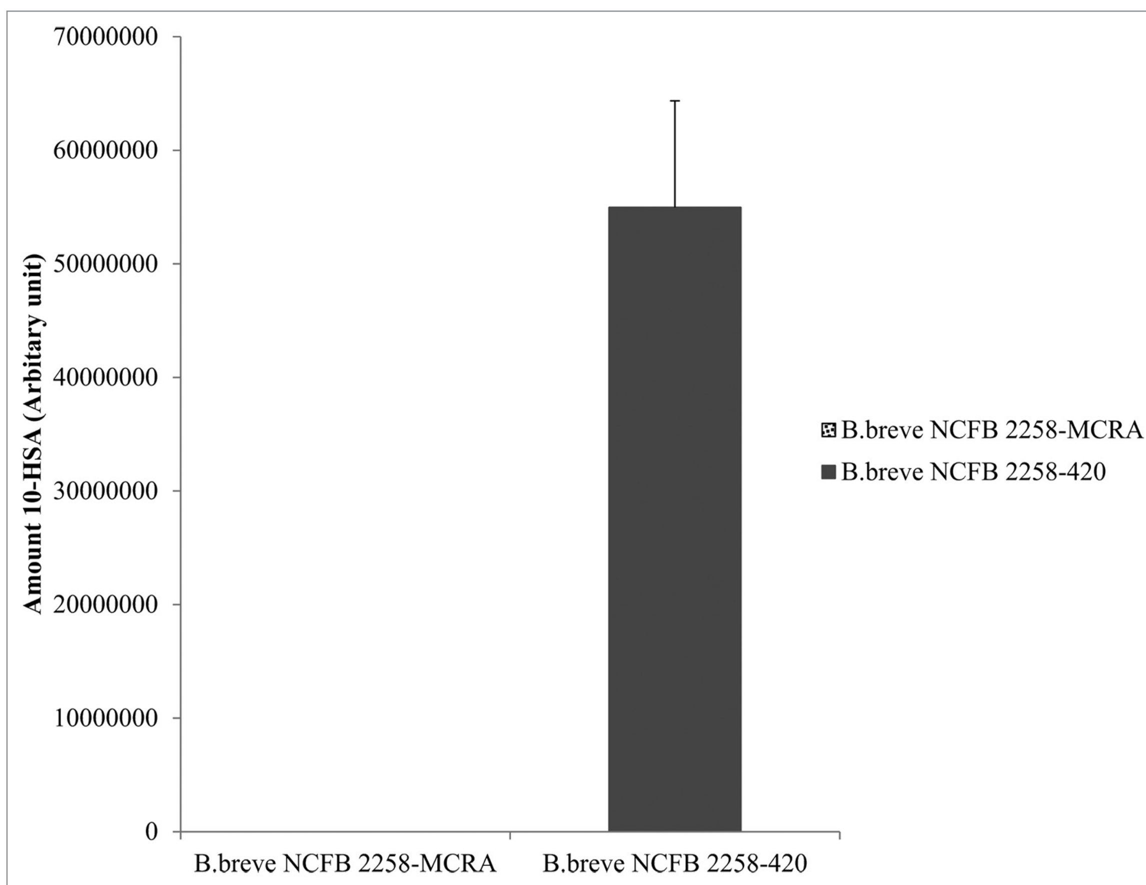

Figure 2. The ability of *B. breve* NCFB 2258-MCRA and *B. breve* NCFB 2258-420 (control) to convert oleic acid to 10-hydroxystearic acid was assayed by incubating cultures in MRS broth which contains 1% oleic acid at 37°C for 16 h, with subsequent assessment of the fatty acid profile.

10-hydroxystearic acid (10-HSA).[11] In order to test if hydratase activity was affected in *B. breve* NCFB 2258-MCRA carrying a mutation in $MCRA_{NCFB2258}$, this strain was subjected to a hydratase activity test as previously described.[20] In contrast to the control strain *B. breve* NCFB 2258-420, which carries an insertion in Bbr_0420, previously characterized as an α-galactosidase,[33] the insertion mutant *B. breve* NCFB 2258-MCRA did not exhibit hydratase activity above the thresh-hold detection level (**Fig. 2**), thus substantiating the hypothesis that the $MCRA_{NCFB2258}$ gene product acts as a hydratase that converts oleic acid into 10-HSA.

MCRA insertion mutation affects the stress tolerance of *B. breve* NCFB 2258. Heterologous expression of the *B. breve* NCFB 2258 MCRA protein in *Lactococcus* and *Corynebacterium* had previously been shown to cause increased stress tolerance to 3% (v/v) butanol.[11] This finding suggests that a mutation in this gene in *B. breve* NCFB 2258 would consequently lead to increased sensitivity to certain stressful conditions. In order to test this assumption, *B. breve* NCFB 2258-MCRA and *B. breve* NCFB 2258-420 (control) were grown to an OD600 nm of between 0.4 and 0.5, prior to exposure to solvent stress, which was achieved by the addition of ethanol (final concentration 16%; v/v). The obtained results show that the *B. breve* NCFB 2258-MCRA insertion mutant is indeed more sensitive to solvent stress than the control strain *B. breve* NCFB 2258-420 in the presence of ethanol (**Fig. 3**).

Discussion

The aim of the current study was to investigate the biological function of the MCRA-encoding gene of *Bifidobacterium breve* in the hydration of oleic acid into 10-hydroxyoctadecanoic acid and its possible role in CLA production. We showed that various bifidobacterial strains have different CLA production capabilities with *B. breve* NCFB 2258 and *B. breve* JCM 7017 producing high levels of CLA[30,34,35] while the tested *B. longum* strains did not produce measurable amounts of CLA from LA, a feature common among *B. longum* strains.[34,36] Interestingly, analysis of the CLA-producing capability of *B. breve* NCIMB 8807 showed that this strain lost this ability during repeated sub-cultivation, a phenomenon that has not been reported previously.

In this study we demonstrated that the MCRA-encoding gene from *B. breve* NCFB 2258 is an oleate hydratase, which is responsible for the breakdown from oleic acid to 10-hydroxystearic acid. MCRA-encoding genes from various bacteria, including bifidobacteria, have been associated with hydratase activity.[16,20,37,38] The MCRA-like protein has been implicated in the first step of CLA production, an assumption which is based on the finding that CLA production from LA by *L. acidophilus* is presumed to be a two-step process in which hydroxy fatty acids act as intermediates in the production of CLA.[28] This hypothesized mechanism

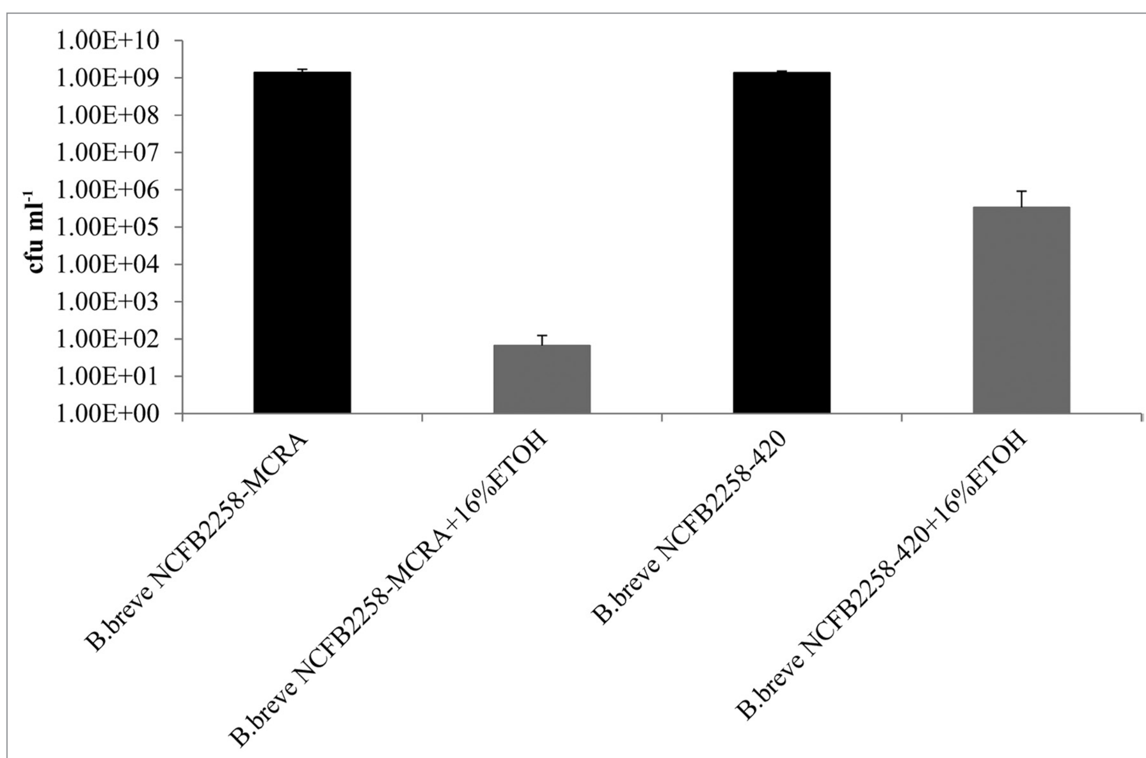

Figure 3. *B. breve* NCFB 2258-MCRA and *B. breve* NCFB 2258–420 (control) grown in the absence and presence of ethanol 16% (v/v). Log phase cells were grown to an OD600 nm of 0.4–0.5 prior to stress of ethanol 16% (v/v), after 180 min cultures were spot plated and incubated at 37°C for 48 hrs anaerobically, this was followed by viable cell counts. The values represent the average of three independent experiments with standard error.

of bacterial CLA production was based on the finding that washed cells of the strain *Lb. acidophilus* AKU 1137 were shown to rapidly convert hydroxy fatty acids to their respective CLA isomers. Therefore CLA production by *Lb. acidophilus* was proposed to involve hydration of LA to 10-hydroxy-18:1, followed by the dehydration and isomerisation of hydroxy fatty acids to *c*9,*t*11 CLA. It is evident in this study that the MCRA-encoding gene of *B. breve* NCFB 2258 is not involved in CLA production and that bifidobacteria follow a different biochemical route to achieve LA to CLA conversion. It is also interesting to note that this MCRA gene is present in *B. longum* strains which do not produce CLA such as in this case KJOC1.

In relation to solvent stress, *B. breve* NCFB 2258-MCRA was shown to be more sensitive to 16% ethanol (v/v) as compared with the control strains *B. breve* NCFB 2258 and *B. breve* NCFB 2258–420. In a previous study[37] increased stress tolerance to butanol was observed when the MCRA-like protein from *B. breve* NCFB 2258 was heterologously expressed in *L. lactis* and *C. glutamicum*. Ethanol was chosen as an alternative membrane stress to butanol as similarities exist in how microbes respond to these membrane stress-invoking compounds.[39] The effects of ethanol are a result of dielectric, polar and hydrogen bond interactions with the surface groups of the membrane and membrane proteins, ethanol therefore has the same effects as butanol.[40] Previous studies have suggested that MCRA proteins play a role in stress tolerance within the gastrointestinal tract. Deletion of the MCRA-encoding gene in *L. acidophilus* was shown to result in a strain that exhibited reduced growth in the presence of lactate, acetate and salt, which is consistent with a reduced membrane integrity.[41]

Ethanol and butanol are short chain alcohols and bacteria respond to these compounds in a similar manner through changes in membrane fatty acid composition, structure and membrane fluidity.[39] In gram-negative bacteria exposure to ethanol leads to disruption of cell membrane integrity and structure which then leads to cell death. Ethanol-tolerant phenotypes in some *E. coli* strains are known to result from adaptive changes in the composition of the membrane fatty acids with fatty acid length changing from 16 to 18 carbons, while other changes in the composition of the cell envelope may also confer tolerance.[39]

The mechanism underlying gram-positive bacterial tolerance to organic solvents has not been studied extensively,[42] and have been speculated to be due to the induction of a general stress regulon,[43] production of organic solvent-emulsifying or deactivating enzymes[44] and/or an active solvent efflux pump.[44,45] Unsaturated fatty acids are toxic for many bacteria due to their detrimental effect on the cytoplasmic membrane and it has been hypothesized that MCRA ensymes may provide a detoxification mechanism by the hydration of unsaturated fatty acids.[20] This detoxification activity may also apply to the bifidobacterial MCRA proteins and may represent a physiological adaptation to the colonic environment.

Collectively our results demonstrate that the MCRA-like protein encoded by *B. breve* NCFB 2258 is an oleate hydratase, which plays a role in stress tolerance in bifidobacteria, while it does not appear to have a role in LA to CLA conversion by *B. breve*. The

Table 2. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
E. coli strains		
EC101	Cloning host, repA⁺ km^r	52
B. breve strains		
UCC2003	Isolate from a nursling stool	54
NCIMB 8807	Isolate from a nursling stool	NCIMB
NCFB 2257	Isolate from infant intestine	NCFB
NCTC 11815	Isolate from infant intestine	NCTC
NCFB2258	Isolate from infant intestine	NCFB
NCIMB 8815	Isolate from infant feces	NCIMB
JCM 7017	Isolate from human feces	JCM
UCC2005	Isolate from human feces	UCC
UCC2007	Isolate from human feces	UCC
NIZO658	Isolate from a nursling stool	NIZO
LMG 13208	Isolate from infant intestine	LMG
UCC1	Isolate from human feces	UCC
NCFB2258-MCRA	pORI19-tet-MCRA insertion mutant of 2258	This study
NCFB2258-420	pORI19-tet-420 insertion mutant of 2258	This study
B. longum strains		
UCC2	Isolate from human feces	UCC
UCC3	Isolate from human feces	UCC
KJOC1	Isolate from human feces	UCC
KJOC2	Isolate from human feces	UCC
Plasmids		
pORI19	Em^r, repA⁻, ori⁺, cloning vector	52
pAM5	pBC1-puC19-Tc^r	53

JCM, Japan Collection of Microorganisms; NIZO, Nizo Food Research; LMG, Belgian coordinated Collection of Microorganisms; NCFB, National Collection of Food Bacteria; NCIMB, National Collection of Industrial and Marine Bacteria; NCTC, National Collection of Type Cultures; UCC, University College Cork Culture Collection.

actual metabolic pathway of CLA production in bifidobacteria is thus still elusive and our future research efforts will aim to uncover the genetic elements involved in this bioconversion.

Materials and Methods

Bacterial strains, plasmids and culture conditions. Bacterial strains and plasmids used in this study are detailed in **Table 2**. Bifidobacterial strains were routinely cultured in either de Man Rogosa and Sharpe medium (MRS; Difco™, BD, 288130) supplemented with 0.05% cysteine-HCl or reinforced clostridial medium (RCM; Oxoid Ltd. CM0149). Bifidobacterial cultures were incubated at 37°C under anaerobic conditions which were maintained using an Anaerocult oxygen depleting system with an atmosphere of 5% CO_2-5% H_2-90% N_2 (Merck, 1.13829.0001) in an anaerobic chamber. *Escherichia coli* strains were cultured in Luria-Bertani broth (LB)[46] at 37°C with agitation, where appropriate growth media contained erythromycin (Em; 100 μgml^{-1} for *E. coli*), tetracycline (Tet; 10 μgml^{-1} for *E. coli* and *B. breve*) or kanamycin (Km; 50 μgml^{-1} for *E. coli*).

Nucleotide sequence analysis. Sequence data were obtained from the Artemis-mediated[47] genome annotations of the *B. breve* UCC2003 genome.[32] Database searches were performed using non-redundant sequences accessible at the National Centre for Biotechnology Information internet site (http://www.ncbi.nlm.nih.gov) using Blast.[48,49] Sequence assembly, verification and analysis were performed using the Seqman and Seqbuilder programs of the DNASTAR software package (DNASTAR, v10.1.2).

DNA manipulations. Chromosomal DNA was isolated from bifidobacteria as previously described.[50] Minipreparation of plasmid DNA from *E. coli* was achieved using the Qiaprep spin plasmid miniprep kit (Qiagen GmBH, 11754785001). Procedures for DNA manipulations were performed essentially as previously described.[46] Restriction enzymes and T4 DNA ligase were used according to the supplier's instructions (Roche Diagnostics, C126A). Synthetic single stranded oligonucleotide primers used in this study (**Table 3**) were synthesized by MWG Biotech AG. Standard PCRs were performed using TaqPCR mastermix (Qiagen 201445), *B. breve* colony PCRs were performed using reddymix extensor PCR mastermix (Thermo scientific AB-0794-B), and both of these PCR procedures were performed according to manufacturer's instructions using a biometra T3000 thermocycler (Biometra GmbH, Rudolf-Wissell-Str. 30, D-37079). PCR amplicons were purified using the Qiagen PCR purification kit (Qiagen, 11732676001). Electroporation of plasmid DNA into *E. coli* or *B. breve* NCFB 2258 was performed as previously described.[46,51] The correct orientation and integrity of all constructs was verified by DNA sequencing, performed at MWG Biotech.

Construction of *B. breve* insertion mutant. *B. breve* NCFB 2258 is highly transformable achieving transformation efficiencies comparable to those achieved with *B. breve* UCC2003 and it was observed that methylation of plasmid DNA in *E. coli* pNZ-MBbrI-MBbrII allows homologous recombination to be achieved in *B. breve* NCFB 2258. An internal fragment of the putative MCRA-encoding gene Bbr_1293 (472 bp) was amplified by PCR using *B. breve* UCC2003 chromosomal DNA as template and primer combination MCRAFhd3 and MCRARxba1 (**Table 3**). *B. breve* UCC2003 sequence was used to design all primers as the *B. breve* NCFB 2258 genome sequence is unavailable. The generated PCR product was ligated to pORI19, an Ori⁺ RepA⁻ integration plasmid,[52] using HindIII and XbaI restriction sites that were incorporated into the primers for the MCRA amplicons and introduced into *E. coli* EC101 by electroporation. Recombinant *E. coli* EC101 derivatives containing pORI19-based constructs were selected on LB agar containing Em and supplemented with X-gal (5-bromo-4-chloro-3-indolyl-D-galactopyranoside) (40 gml^{-1}) and 1 mM IPTG.

The expected genetic structure of the recombinant plasmid, designated pORI19-MCRA (pORI19 containing an internal 472 bp fragment of the MCRA-encoding gene), was confirmed by restriction mapping prior to subcloning of the Tet resistance

Table 3. Oligonucleotide primers used in this study

Purpose	Primer	Sequence[a]	Size
Amplification of MCRA internal fragment from *B. breve* UCC2003 to create mutant	MCRAFhd3 MCRARxba1	TGC ATC **AAG CTT** CGA TGA CGA GGT GCT GAA C TGC GCA **TCT AGA** CAG CCG CCG TTG GTG ATG	472 bp
Confirmation of MCRA integration in *B. breve* NCFB 2258	MCRA -confirm Tetwsal1F	CTA CAG CAG CGG CAA CTA TG TCA GCT **GTC GAC** ATG CTC ATG TAC GGT AAG gtt gct act ccc tct gac tct cc TCA GCT **GTC GAC** ATG CTC ATG TAC GGT AAG	3.5 KB
Confirmation of 420 integration in *B. breve* NCFB 2258	420-confirm Tetsal1F	GTT GCT ACT CCC TCT GAC TCT CC TCA GCT **GTC GAC** ATG CTC ATG TAC GGT AAG	3.2 KB
Confirmation of tetw integration in *B. breve* NCFB 2258	Tetwsal1F Tetwsal1R	TCA GCT **GTC GAC** ATG CTC ATG TAC GGT AAG GCG ACG **GTC GAC** CAT TAC CTT CTG AAA CAT A	2.2 KB

[a]Sequences of restriction enzyme sites are indicated in bold.

antibiotic cassette, *tetW*, from pAM5[53] as a SacI fragment into the unique SacI site of pORI19-MCRA.

The orientation of the tetracycline resistance gene in the resulting plasmid, designated pORI19-tet-MCRA, was determined by restriction analysis. The plasmid was subsequently introduced into *E. coli* EC101 pNZ-MBbrI-MBbrII and transformants were selected based on Cm and Tet resistance. Methylation of the plasmid complement of the obtained transformants in EC101 pNZ-MBbrI-MBbrII was confirmed by their observed resistance to PstI restriction.[54] The methylated pORI19-tet-MCRA plasmid was introduced into *B. breve* NCFB 2258 by electroporation, with subsequent selection for tetracycline resistance on RCA plates supplemented with Tet. The methylated pORI19-tet-420 plasmid[33] was introduced into *B. breve* NCFB 2258 by electroporation, with subsequent selection for tetracycline resistance on RCA plates supplemented with Tet.

Insertion mutants resulting from site specific homologous recombination were initially confirmed by colony PCR targeting the tetracycline resistance gene *tetW*. This was followed by a second confirmatory PCR adopting a *tetW*-based primer, either forward or reverse depending on the orientation of *tetW*, in combination with a primer specific for each targeted gene to confirm integration at the correct chromosomal position. In this case a product would only be obtained if the correct gene disruption had been achieved.

Rapid analysis method for conjugated linoleic acid production. The ability of isolates to convert free linoleic acid to CLA was assayed by incubating cultures in mMRS[55] broth supplemented with free linoleic acid (0.5 $mgml^{-1}$) (Sigma, L1379) at 37°C for 72 h, with subsequent assessment of the fatty acid profile using a rapid detection method for CLA production.[29] A standard curve[29] demonstrated that an increase in the CLA concentration (from 0 to 0.05 mg/ml) coincided with a linear increase (R^2 = 0.9985) in absorbance for the *c*9,*t*11 CLA isomer up to an absorbance of 2.1. Therefore, the CLA concentrations in culture supernatants with an absorbance at 233 nm less than or equal to 2.1 could be calculated from the linear trend line of the standard curve using the equation $y = 43.431x + 0.0053$.

Gas liquid chromatography (GLC) method for conjugated linoleic acid production. The ability of isolates to convert free linoleic acid to CLA was assayed by incubating cultures in mMRS broth supplemented with free linoleic acid (0.5 $mgml^{-1}$) at 37°C for 72 h. Fatty acids were extracted from four grams of the fermented sample following addition of 0.75 mg of the internal standard tridecanoic acid (Sigma Aldrich 91988). Two milliliters of isopropanol (99% purity, Labscan, PLA19_X) and four milliliters of n-hexane (Labscan, PLA08_X) were added to the sample followed by vortexing for two min. Following centrifugation at 2197 g for 5–6 min, the resulting clear upper layer was removed to a clean glass tube and evaporated by heating at 45°C under a steady flow of nitrogen. Extracted fatty acids were converted to fatty acid methyl esters (FAMEs) by acid catalyzed methylation using 4% methanolic HCl (Supleco, 33050-U) at 60°C for 20 min and analyzed by GLC analysis.[30,56]

Determination of hydratase activity. Determination of hydratase activity was performed as previously published,[11] with the following modification: before extraction bacterial cells were ground into a fine powder under liquid nitrogen using a beat mill from Retsch and stored at -80°C. GC/MS-analysis was then performed as indicated in a previous publication.[20]

Stress tolerance. *B. breve* NCFB 2258-MCRA and *B. breve* NCFB 2258–420, which carries a mutation in a gene previously shown to encode a α-galactosidase (control),[33] were grown to OD600 nm 0.4–0.5, prior to stress. Solvent tolerance was determined on addition of ethanol (final concentration 16%; v/v), after 180 min ten microliter volumes of serially diluted samples were spotted in triplicate on reinforced clostridial agar plates. Plates were incubated for 48 h at 37°C anaerobically. Spots containing between 3 and 30 colony forming units CFU were counted and the average was used to calculate the number of CFU per milliliter. The values presented are averages of triplicate experiments.

Disclosure of Potential Conflicts of Interest

No conflicts of interest were disclosed.

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