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. Bifidobacteria were first isolated more than a century ago and naturally inhabit the gastrointestinal tract of humans and other mammals, being particularly abundant in breast-fed infants. Bifidobacteria have been claimed to maintain gastrointestinal health and are therefore used as a health-promoting or probiotic bacterial ingredients in certain functional foods. Their reported beneficial effects on the host include inhibition of pathogens, alleviating lactose intolerance, enhancing natural immunity and reducing serum cholesterol.

Bifidobacteria have been shown to convert oleic acid (OA) to 10-hydroxystearic acid (10-HSA). OA is a mono-unsaturated fatty acid that occurs naturally in vegetable oils and is reported to confer a range of health benefits including the inhibition of adenine deoxynucleotidase (ALD), a metabolic disorder that leads to demyelination in the central and peripheral nervous system and reductase in blood pressure. 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. However, the isolation and biochemical characterization of the enzyme responsible for this conversion, the oleate hydratase, was not reported until relatively recently. The latter authors also showed that expression of the El. 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, which is probably due to the disruptive effect of these hydrophobic compounds on the bacterial membrane, combined with their inhibitory action on enoyl-ACP reductase (FabH) and thus fatty acid biosynthesis. 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.

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. 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. 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.

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MCRA-like proteins show over 50% sequence similarity to the linoleic acid isomerase (LAI) from Lactobacillus acidophilus and Lb. reuteri PVR8. LAI catalyzes the conversion of linoleic acid (LA, a dienoic unsaturated fatty acid) to cis-9 trans-11 (c9,t11) conjugated linoleic acid (CLA). CLA is a collective term used to describe a group of polyunsaturated fatty acids that exist as positional and geometric isomerisms of octadecadienoic acid. Twenty-eight different CLA isomers have been identified, of which the most abundant is the c9,t11 isomer, representing approximately 80% of total CLA in food products. CLA is found naturally in ruminant food products, e.g., lamb, beef and dairy, due to the process of biohydrogenation of LA in the rumen. LAI protein is a membrane-bound enzyme which was first isolated from Butyrivibrio fibrisolvens. Subsequently, the crystal-structure of an LAI from Propionibacterium acne was solved and the reaction mechanism characterized. It was recently suggested 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. 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. 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 was adopted. Our results demonstrate that B. breve NCFB 2258 was the strain with the highest CLA (c9,t11) 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.

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

| Strain          | CLA converted from 0.5mg ml\(^{-1}\) Linoleic acid\(^{a}\) | Source            | \(\%\) Converted |
|-----------------|-------------------------------------------------------------|-------------------|-----------------|
| Bifidobacterium breve |                                             | Source            | \(\%\) Converted |
| UCC2003         | 0.008                                                       | Isolate from a nursing stool | 1.73            |
| NCIMB 8807      | 0.085                                                       | Isolate from a nursing stool | 17.16           |
| NCFB 2257       | 0.027                                                       | Isolate from infant intestine | 5.54            |
| NCTC 11815      | 0.148                                                       | Isolate from infant intestine | 29.64           |
| NCFB 2258       | 0.245                                                       | Isolate from infant intestine | 49.00           |
| NCIMB 8815      | 0.077                                                       | Isolate from infant feces   | 15.54           |
| KJOC2           | 0.229                                                       | Isolate from human feces    | 45.90           |
| UCC2005         | 0.179                                                       | Isolate from human feces    | 35.88           |
| UCC2007         | 0.014                                                       | Isolate from human feces    | 10.64           |
| Nizo 658        | 0.083                                                       | Isolate from a nursing stool | 16.70           |
| LMG 13208       | 0.055                                                       | Isolate from infant intestine | 11.16           |
| UCC1            | 0.005                                                       | Isolate from human feces    | 1.05            |
| NCFB 2258-MCRA  | 0.213                                                       | This study            | 42.60           |

*Values represent the average of two independent experiment.
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 \textit{B. breve} NCFB 2258. As mentioned above, MCRA-like proteins exhibit over 50% sequence similarity to LAI from \textit{Lactobacillus acidophilus} and \textit{Lb. reuteri} PYR8,22 which have been implicated in the conversion of LA to \(c_{9},t_{11}\) CLA. It was recently also suggested\textsuperscript{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.\textsuperscript{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 \textit{MCRANCFB2258}, of \textit{B. breve} NCFB 2258 after which the CLA production capability of the mutant strain was analyzed. Based on the rapid spectrophotometer method of\textsuperscript{29} the \textit{B. breve} NCFB 2258-MCRA insertion mutant was shown to produce the same amount of CLA as the parent strain \textit{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 \textit{B. breve} NCFB 2258, at least under the conditions tested.

Identification and analysis of a \textit{B. breve} UCC2003 myosin cross reactive encoding gene. A gene designated \textit{MCRA\textsubscript{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 \textit{B. breve} UCC2003.\textsuperscript{30} At protein level, the predicted product of \textit{MCRA\textsubscript{ucc2003}} is almost identical (99% identity) to predicted MCRAs encoded by homologs on the genomes of \textit{Bifidobacterium breve} CETC 7263 (GenBank accession number ADY18551.1), DSM 20213 (GenBank accession number 2ZP_12586529.1), ACS-071-V-Sch8b (GenBank accession number 2ZP_0696930.1), 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 \textit{B. animalis} subsp. lactis HN019 (GenBank accession number ZP_02963377) and \textit{B. animalis} subsp. \textit{animalis} ATCC 25527 (GenBank accession number YP_006279649.1), where just 68% identity is observed compared with \textit{MCRA\textsubscript{ucc2003}}. The apparently monocistronic \textit{MCRA\textsubscript{ucc2003}} (1,878 bp) on the \textit{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 \textit{B. breve} NCFB 2258. As mentioned above, MCRA-like proteins exhibit over 50% sequence similarity to LAI from \textit{Lactobacillus acidophilus} and \textit{Lb. reuteri} PYR8,22 which have been implicated in the conversion of LA to \(c_{9},t_{11}\) CLA. It was recently also suggested\textsuperscript{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.\textsuperscript{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 \textit{MCRA\textsubscript{ucc2003}} of \textit{B. breve} NCFB 2258 after which the CLA production capability of the mutant strain was analyzed. Based on the rapid spectrophotometer method of\textsuperscript{29} the \textit{B. breve} NCFB 2258-MCRA insertion mutant was shown to produce the same amount of CLA as the parent strain \textit{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 \textit{B. breve} NCFB 2258, at least under the conditions tested.

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

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure1.png}
\caption{Schematic representation of the comparison of the myosin cross reactive antigen encoding gene, \textit{MCRA\textsubscript{ucc2003}} from \textit{B. breve} UCC2003 to other sequenced \textit{B. breve}.\textsuperscript{30}}
\end{figure}
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, while the tested B. longum strains did not produce measurable amounts of CLA from LA, a feature common among B. longum strains. 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. 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, 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. 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).
reduced growth in the presence of lactate, acetate and salt, which is consistent with a reduced membrane integrity. 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. 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. The mechanism underlying gram-positive bacterial tolerance to organic solvents has not been studied extensively, and have been speculated to be due to the induction of a general stress regulon, production of organic solvent-emulsifying or deactivating enzymes and/or an active solvent efflux pump. Unsaturated fatty acids are toxic for many bacteria due to their detrimental effect on the cytoplasmic membrane and it has been hypothesized that MCRA enzymes may provide a detoxification mechanism by the hydration of unsaturated fatty acids. 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 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 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 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. 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. 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.

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. 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. The mechanism underlying gram-positive bacterial tolerance to organic solvents has not been studied extensively, and have been speculated to be due to the induction of a general stress regulon, production of organic solvent-emulsifying or deactivating enzymes and/or an active solvent efflux pump. Unsaturated fatty acids are toxic for many bacteria due to their detrimental effect on the cytoplasmic membrane and it has been hypothesized that MCRA enzymes may provide a detoxification mechanism by the hydration of unsaturated fatty acids. 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

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.
maintained using an Anaerocult oxygen depleting system with an Anaerocult A (Merck), and were incubated at 37°C under anaerobic conditions which were maintained using an Anaerocult A (Merck), and were incubated at 37°C under anaerobic conditions. Bacterial cultures were supplemented with 0.05% cysteine-HCl or reinforced clostridial medium (RCM; Oxoid Ltd. CM0149). Bifidobacterial cultures were cultured in an anaerobic chamber. Escherichia coli strains were cultured in Luria-Bertani broth (LB) at 37°C with agitation, where appropriate growth media contained erythromycin (Em; 10 μg/ml for E. coli), tetracycline (Tet; 10 μg/ml for E. coli and B. breve) or kanamycin (Km; 50 μg/ml for E. coli).

Nucleotide sequence analysis. Sequence data were obtained from the Artemis-mediated47 genome annotations of the B. breve UCC2003 genome.48 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.46 Mini-preparations of plasmid DNA from E. coli was achieved using the Qiagen spin plasmid mini prep 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, C26A). 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 2014145), E. coli colony PCRs were performed using redyddmix extension PCR mastermix (Thermo scientific AB-0794-B), and both of these PCR procedures were performed according to manufacturer's instructions using a biometra T3001 thermocycler (Biometra GmbH, Rudolf-Wissel-Str. 30, D-37079). PCR amplifications 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 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-MBbr-MBbrI 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’ Rep’ 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 gm/l) 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 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’ | 52                  |
| **B. breve strains** |                         |                     |
| UCC503            | Isolate from a nursling stool | 54                |
| NCMB 8807         | Isolate from a nursling stool | NCMB            |
| NCFB 2257         | Isolate from infant intestine | NCFB       |
| NCTC 11815        | Isolate from infant intestine | NCTC       |
| NCFB258           | Isolate from infant intestine | NCFB       |
| NCMB 8815         | Isolate from infant feces | NCMB               |
| JCM 2017          | Isolate from human feces | JCM                |
| UCC2005           | Isolate from human feces | UCC                |
| UCC2007           | Isolate from human feces | UCC                |
| NIZD658           | Isolate from a nursling stool | NIZD           |
| LGM 13008         | Isolate from infant intestine | LGM           |
| UCC1              | Isolate from human feces | UCC                |
| **NCFB2258-MCRA** |                         | This study          |
| pORI19-tet-MCRA insertion mutant of 2258 |             |
| **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’, repA’, ori’, cloning vector | 52          |
| pM15             |                         | 53                  |

JCM, Japan Collection of Microorganisms; NCBB, Nizo Food Research; LMG, Belgian coordinated Collection of Microorganisms; NCBF, National Collection of Food Bacteria; NCMB, 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% CO₂, 5% H₂, 90% N₂ (Merek, 113829.0001) in an anaerobic chamber. Escherichia coli strains were cultured in...
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-MBbr1-MBbrII and transformants were selected based on Cm and Tet resistance. Methylation of the plasmid of the obtained transformants in EC101 pNZ-MBbr1-MBbrII was confirmed by their observed resistance to Pol restriction.25 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 plasmid25 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 broth supplemented with free linoleic acid (0.5 mg/ml) (Sigma, L1379) at 37°C for 72 h, with subsequent assessment of the fatty acid profile using a rapid detection method for CLA production.27 A standard curve27 demonstrated that an increase in the CLA concentration (from 0 to 0.05 mg/ml) coincided with a linear increase (R² = 0.9985) in absorbance for the ε9t11 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 = 4.3431x - 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 mg/ml) 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, PLA08_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 (Supelco, 33050-U) at 60°C for 20 min and analyzed by GLC analysis.28,30

Determination of hygratase activity. Determination of hygratase activity was performed as previously published,22 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 n-α-galactosidase (control),27 were grown to ODE600 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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Table 3. Oligonucleotide primers used in this study

| Purpose | Primer | Sequencea | Size |
|---------|--------|-----------|------|
| Amplification of MCRA internal fragment from B. breve UCC11833 to create mutant | MCRAlped3 | TGC ATT AAG CTT CGA TGA CGA GCT GCT GAA C | 472 bp |
| -confirm | MCRABred1 | TGC GCA TCT AGA CAG CCG CCG TIG GTG ATG | |
| Confirmation of MCRA integration in B. breve UCC11833 | MCRA -confirm | CTA CAG CAG CGG CGA CTA TG | 3.5 KB |
| TetralM1F | TCA GCT GTC GAC ATG CTC ATG TAC GAT AAG | |
| Confirmation of tetW integration in B. breve NCFB 2258 | TetralM1F | TCA GCT GTC GAC ATG CTC ATG TAC GAT AAG | 3.2 KB |
| 420-confirm | TetralM1F | TCA GCT GTC GAC ATG CTC ATG TAC GAT AAG | |
| Confirmation of TetralM1F integration in B. breve NCFB 2258 | TetralM1F | TCA GCT GTC GAC ATG CTC ATG TAC GAT AAG | 2.2 KB |
| | TetralM1F | GGC AGC GTC GAC CAT TAC CTC AAA CAT A | |

-Sequences of restriction enzyme sites are indicated in bold.
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