Functional and phenotypic characterization of a protein from *Lactobacillus acidophilus* involved in cell morphology, stress tolerance and adherence to intestinal cells

Sarah J. O'Flaherty and Todd R. Klaenhammer

Correspondence
Todd R. Klaenhammer
klaenhammer@ncsu.edu

Department of Food, Bioprocessing and Nutrition Sciences, North Carolina State University,
Raleigh, NC 27695, USA

Structural components of the cell surface have an impact on some of the beneficial attributes of probiotic bacteria. *In silico* analysis of the *L. acidophilus* NCFM genome sequence revealed the presence of a putative cell surface protein that was predicted to be a myosin cross-reactive antigen (MCRA). As MCRA are conserved among many probiotic bacteria, we used the *upp*-based counterselective gene replacement system, designed recently for use in *L. acidophilus*, to determine the functional role of this gene (LBA649) in *L. acidophilus* NCFM. Phenotypic assays were undertaken with the parent strain (NCK1909) and deletion mutant (NCK2015) to assign a function for this gene. The growth of NCK2015 (Δ LBA649) was reduced in the presence of lactate, acetate, porcine bile and salt. Adhesion of NCK2015 to Caco-2 cells was substantially reduced for both stationary-phase (~45 % reduction) and exponential-phase cells (~50 % reduction). Analysis of NCK2015 by scanning electron microscopy revealed a longer cell morphology after growth in MRS broth compared to NCK1909. These results indicate a role for LBA649 in stress tolerance, cell wall division and adherence to Caco-2 cells.

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INTRODUCTION

Species of the genus *Lactobacillus*, such as *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus helveticus*, have long been used in fermented foods as starter cultures in yogurt and in cheese manufacture, respectively. Others are classified as probiotic bacteria and fall into the definition of a probiotic as defined by the World Health Organization: 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (www.who.int/foodsafety/publications/fs_management/probiotics2/en/). *Lactobacillus acidophilus* is one of the most widely used probiotic species in the US and has demonstrated health benefits, such as lowering levels of cold and influenza-like symptoms during antibiotic administration in children (Leyer *et al.*, 2009) and decreasing the duration of diarrhoea (Liévin-Le Moal *et al.*, 2007). Additional studies with *L. acidophilus* have demonstrated modulation of intestinal pain and induction of pain receptors in intestinal cells which exert analgesic effects in colitis models (Rousseaux *et al.*, 2007), and activation of toll-like receptors and dendritic cells (Konstantinov *et al.*, 2008; Mohamadzadeh & Klaenhammer, 2008).

Understanding probiotic bacteria and their effector molecules that contribute to health-promoting properties has

been facilitated by advances in molecular tools and 'omic' technologies. Since the genome sequence of *L. acidophilus* was published in 2005 (Altermann *et al.*, 2005) there have been numerous studies on the transcriptome of this bacterium under conditions such as acid and bile stress, and carbohydrate metabolism (Azcarate-Peril *et al.*, 2005; Barrangou *et al.*, 2006; Pfeiler & Klaenhammer, 2009; Pfeiler *et al.*, 2007). In addition, gene inactivation or deletion has confirmed the role of genes contributing to probiotic traits, such as bile tolerance (Pfeiler & Klaenhammer, 2009; Pfeiler *et al.*, 2007), acid stress (Azcarate-Peril *et al.*, 2004), transport and catabolism of fructooligosaccharide (Barrangou *et al.*, 2003), oxalate degradation (Azcarate-Peril *et al.*, 2006) and bacteriocin transport (Dobson *et al.*, 2007).

The contribution of cell-surface or cell-surface-displayed factors by probiotic bacteria and their interaction with the pattern recognition receptors of the intestinal cells of the gastrointestinal tract is the initial step in initiation and modulation of the immune response (Grangette *et al.*, 2005; Konstantinov *et al.*, 2008; reviewed by Lebeer *et al.*, 2010). These surface molecules are varied and interact with different receptors, resulting in diverse responses from the host. Importantly these interactions and the resulting responses are varied between probiotic, commensal and pathogenic bacteria (Boneca *et al.*, 2007; Fischer *et al.*, 2006; Grangette *et al.*, 2005; Konstantinov *et al.*, 2008).

Abbreviation: MCRA, myosin cross-reactive antigen.

Cell-surface molecules from *L. acidophilus* NCFM have been inactivated or deleted, and the mutant strains subsequently demonstrated reduced adherence to intestinal cell lines (Buck *et al.*, 2005) and modulation of dendritic and T cell functions (Konstantinov *et al.*, 2008).

Myosin cross-reactive antigens (MCRAs) are conserved proteins found across a wide range of taxa, including lactic acid bacteria (Volkov *et al.*, 2010). The annotation MCRA that is conferred to these conserved proteins is derived from their initial discovery as potential antigens of *Streptococcus pyogenes* that shared epitopes with myosin (Kil *et al.*, 1994). However, recently an MCRA from *S. pyogenes* was demonstrated to have hydratase activity and to contribute to blood survival and adherence to keratinocytes (Volkov *et al.*, 2010). *In silico* analysis of the *L. acidophilus* NCFM genome sequence revealed the presence of a gene annotated as an MCRA. As MCRAs are conserved among many probiotic bacteria, we used the *upp*-based counterselective gene replacement system, designed recently for use in *L. acidophilus* (Goh *et al.*, 2009), to construct a strain of *L. acidophilus* NCFM with a deletion in the LBA649 gene. The resulting mutant strain was compared to the parent strain to determine a function for this gene. Results indicated that LBA649 plays a role in adherence to Caco-2 cells, stress tolerance and cell morphology.

METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *L. acidophilus* strains were propagated statically at 37 °C under ambient atmospheric conditions in de Man, Rogosa and Sharpe (MRS; Difco) broth. *Escherichia coli* strains were grown in brain heart infusion (BHI;

Difco) broth at 37 °C with aeration. Solid media contained 1.5 % (w/v) bacteriological agar (Difco). *E. coli* EC101 was propagated in the presence of 40 µg kanamycin ml⁻¹ and, when necessary, chloramphenicol and erythromycin were added at final concentrations of 15 and 200 µg ml⁻¹, respectively. Recombinant *L. acidophilus* strains were selected as described previously (Goh *et al.*, 2009).

DNA manipulations and sequence analysis. Standard procedures were used for molecular manipulations (Sambrook & Russel, 2001). Genomic DNA was isolated from *L. acidophilus* NCFM using the Zymoclean Fungal/Bacterial DNA kit (Zymo Research Corp.). Plasmid DNA from *E. coli* was isolated using the QIAprep Spin Miniprep kit (Qiagen). Electrocompetent *L. acidophilus* cells were prepared as described by Walker *et al.* (1996) and *E. coli* competent cells were prepared as described by Hanahan (1985). Restriction enzymes (Roche Molecular Biochemicals) and T4 ligase (New England Biolabs) were used according to the manufacturers' instructions. The Expand High-Fidelity PCR system (Roche Molecular Biochemicals) was used for cloning purposes and Choice-*Taq* Blue DNA polymerase (Denville Scientific Inc.) was used for screening recombinants. PCR products were purified using the Zymoclean Gel DNA Recovery kit (Zymo Research Corp.) or the QIAquick Gel Extraction kit (Qiagen). PCR primers (Table 1) were purchased from Integrated DNA Technologies and sequencing was performed by Davis Sequencing Inc. and Genewiz.

Sequence analysis was performed using Clone Manager 6.0 (Scientific and Educational Software). Protein sequences were searched against the protein database using BLAST algorithms (Altschul & Lipman, 1990). Signal peptides were predicted using the SignalP 3.0 (Bendtsen *et al.*, 2004). InterProScan was used to determine conserved protein domains (Quevillon *et al.*, 2005). Sequences were manually searched for the presence of LPXTG motifs, and TransTermHP was used to find rho-independent transcription terminators (Kingsford *et al.*, 2007). The web-based transmembrane prediction programs, TMPRED (www.ch.embnet.org/index.html), HMMTOP (Tusndy & Simon, 2001), TOPPED (Claros & von Heijne, 1994), DAS (Cserz *et al.*, 2002) and TMHMM (Krogh *et al.*, 2001), were used to predict transmembrane domains.

Table 1. Strains, plasmids and primers used in this study

Strain, plasmid or primer	Description*	Reference
Strains		
<i>L. acidophilus</i> NCK1909	Control strain for deletions (NCFM with <i>upp</i> deletion)	Goh <i>et al.</i> (2009)
<i>L. acidophilus</i> NCK1910	NCK1909 harbouring pTRK669	Goh <i>et al.</i> (2009)
<i>L. acidophilus</i> NCK2015	NCK1909 with a deletion in the LBA649 gene	This study
<i>E. coli</i> NCK1831	<i>E. coli</i> (EC101) host for pORI-based plasmids	Law <i>et al.</i> (1995)
Plasmids		
pTRK935	Counterselective integration vector with a <i>upp</i> expression cassette	Goh <i>et al.</i> (2009)
pTRK970	pTRK935 with a modified copy of the LBA649 gene	This study
pTRK669	Ori (pWV01), Cm ^r , Rep ⁺	Russell & Klaenhammer (2001)
Primers for construction of deletions		
LBA649_1	GATCGAGCTCAATAATCCGGTTGATCCAGTT	This study
LBA649_2	ATCTGCAAATGCTTCGTAAT TACCAT	This study
LBA649_3	ATTACGAAGCATTTCGAGATAAGACTTGGGTAGAAGAAC	This study
LBA649_4	GATCGGATCCTAAGAAGGTGACAGAGTGAAC	This study
Primers for PCR and sequencing analysis		
LBA649F	GGTTAATCATAAACGAAGATATAC	This study
LBA649R	TAGGAAATAGATCATGACCTTATAG	This study

*Italicized letters in the primer sequences represent sites for *Sac*I (GAGCTC) and *Bam*HI (GGATCC).

Construction of mutants using the *upp*-based counterselective gene replacement system. Deletions were constructed using the *upp*-based counterselective gene replacement system, designed recently for deleting genes from *L. acidophilus* (Goh *et al.*, 2009). Briefly, an in-frame deletion of the LBA649 gene was constructed by amplifying the 819 and 778 bp flanking regions of LBA649 using oligonucleotide pairs LBA649_1 and LBA649_2, and LBA649_3 and LBA649_4 (Table 1). These purified regions were joined by splicing using overlap extension PCR, digested with *SacI* and *Bam*HI, and subsequently ligated into the *SacI* and *Bam*HI sites of pTRK935 (a pORI-based counterselective integration vector). Ligation mixtures were transformed into *E. coli* EC101 and constructs were confirmed by sequencing. Subsequently, pTRK970 which carried the flanking regions of LBA649 was electroporated into NCK1910 (Table 1). NCK1910 harbours the helper plasmid pTRK669 which provides *repA in trans* for the replication of pTRK970 (a pORI-based plasmid) (Russell & Klaenhammer, 2001). Procedures to isolate double recombinants carrying the deleted allele of LBA649 were performed as described previously using the *upp*-based counterselective gene replacement system (Goh *et al.*, 2009). The advantage of the *upp*-based system is that it utilizes a selectable marker, *upp* (encoding uracil phosphoribosyltransferase), which allows rapid screening of excision recombinants.

Growth assays. Strains were grown in MRS broth to early exponential phase (OD_{600} 0.25–0.30) and subsequently inoculated (1%) into MRS broth containing 0.02% (w/v) SDS or 0.5% (v/v) Triton X-100 for 24 h. Cell numbers were enumerated by plating on MRS agar after 24 h growth at 37 °C under ambient atmospheric conditions. Strains were inoculated (1%) and grown for 24 h in MRS broth and MRS broth containing 1.8% (v/v) sodium lactate (Fisher Scientific), 2% (w/v) sodium acetate (Fisher Scientific), 2% (w/v) NaCl (Fisher Scientific), 0.2% (w/v) porcine bile (Sigma) and 0.5% (w/v) oxgall (Difco) for growth curves in microtitre plates. The OD_{600} was recorded every hour over a 24 h period using a microtitre plate reader (FLOUStar Optima; BMG Technologies).

Scanning electron microscopy (SEM) analysis. Strains were grown in MRS broth to early exponential phase (OD_{600} 0.25–0.3) and subsequently inoculated (1%) into MRS broth and grown for 24 h. Samples were centrifuged for 10 min at room temperature at 3166 g and resuspended in 1:1 (v/v) fixative containing 6% aqueous glutaraldehyde and 0.2 M sodium cacodylate. Samples were processed and scanned with a JOEL JSM 5900LV scanning electron microscope at 15 kV by the Center for Electron Microscopy at North Carolina State University, USA.

Adherence assays with Caco-2 cell lines. The Caco-2 cell line ATCC HTB-37 was purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were used between passage number 23 and 40. All reagents used in maintenance of Caco-2 cells were obtained from Gibco (Invitrogen). Cells were routinely grown in a 95% air/5% CO_2 atmosphere in minimum essential medium (MEM) supplemented with 20% heat-inactivated fetal bovine serum, 0.10 mM non-essential amino acids, 1.0 mM sodium pyruvate and antibiotics (100 mg penicillin G ml^{-1} , 100 mg streptomycin sulfate ml^{-1} and 0.25 mg amphotericin ml^{-1}). Caco-2 cells were seeded at 1.7×10^4 cells cm^{-2} and the medium was changed every 2 days for 21 days to ensure the cells had differentiated completely. On the day of the assay, Caco-2 cells were washed twice with 1 ml PBS, and 1 ml MEM medium without the antibiotics was added to each well. Bacterial cells were grown to stationary phase (16 h) or exponential phase (OD_{600} ~0.6) at 37 °C, washed once with PBS and resuspended in PBS to a final concentration of approximately 2×10^8 c.f.u. ml^{-1} (enumerated by plating on MRS agar plates). One millilitre of the bacterial suspension was added to each well of the Caco-2 monolayer and incubated at 37 °C for 1 h. The monolayers were then washed

five times to remove any unbound bacteria prior to the addition of 1 ml 0.05% (v/v) Triton X-100. Bacterial numbers were enumerated by serial dilution in $0.1 \times$ MRS broth and plating on MRS agar plates.

RESULTS

In silico analysis of LBA649

In silico analysis of the *L. acidophilus* NCFM genome sequence (Altermann *et al.*, 2005) revealed that the LBA649 gene is flanked by two putative terminators, indicating that this gene is transcribed as a monocistronic mRNA transcript. Additionally, the regions up and downstream of the LBA649 gene share a conserved gene order with other lactobacilli, for example *Lactobacillus ultunensis* which was isolated from the stomach mucosa (Roos *et al.*, 2005). The gene order is less conserved at the downstream region in *Lactobacillus gasseri* where there appears to be a loss of the *pacL* gene, which encodes an ATPase in *L. acidophilus* (Fig. 1). While the gene order was similar around homologues in *Lactobacillus*, this was not the case with *Streptococcus pyogenes* and *Staphylococcus aureus* where there was no conservation of gene order (Fig. 1).

The LBA649 gene encodes a protein of 591 aa (GenBank accession number YP_193559) with a calculated size of 67 kDa. *In silico* analysis for cell-wall-sorting conserved sequences revealed that no LPXTG motif or cleavable signal peptide could be determined for the predicted protein. LPXTG is a specific C-terminal anchoring motif that is recognized by the enzyme sortase. Sortase cleaves proteins at the LPXTG motif and covalently links them to peptidoglycan, thereby anchoring these proteins to the cell wall. Analysis of the deduced amino acid sequence with transmembrane prediction programs revealed different results depending on the program used. The TMHMM and DAS programs did not predict a transmembrane domain for LBA649. However, a putative N-terminal transmembrane domain was identified between amino acid positions 25 and 42 using the programs HMMTOP, TOPPED and TMPRED. As these predictions are not based on experimental data, we propose that LBA649 may have a putative transmembrane domain and could be possibly displayed on the cell surface via an N-terminal anchor motif (Bth *et al.*, 2005).

Analysis of the deduced protein sequence indicated that the protein encoded by the LBA649 gene contains the conserved domain COG4716S, which is annotated as an MCRA domain. Despite this annotation, the function of this conserved protein in lactobacilli is unknown. This domain encompasses the whole protein with sequence homology observed throughout the whole domain rather than confined to the N or C terminus. In addition, the analysis indicated similarity at the N-terminal region to a FAD/NAD(P)-binding domain.

Protein BLAST analysis of LBA649 against the non-redundant database (NCBI), demonstrated high sequence homology (99% identity) to a protein annotated as a

Fig. 1. Schematic of the genome region surrounding the *LBA649* gene in *L. acidophilus* NCFM and homologues in *L. ultunensis* DSM 16047, *L. gasseri* ATCC 33323, *Streptococcus pyogenes* M49 591 and *Staphylococcus aureus* Mu50. The *LBA649* gene and corresponding homologues are indicated by the large arrow. Genes that have homologues amongst the strains are linked by straight lines. Numbers on the left and right represent the location of these genes on the chromosome.

linoleate isomerase from *L. acidophilus*. A high level of identity (74–94 %) was also observed to MCRAs from *L. ultunensis* (94 %), *Lactobacillus crispatus* (93 %), *L. helveticus* (93 %), *L. gasseri* (83 and 84 %), *Lactobacillus johnsonii* (83 %) and *Lactobacillus reuteri* (74 and 75 %), demonstrating that this protein is highly conserved. BLAST analysis also revealed a second protein, *LBA555*, annotated as an MCRA in the genome of *L. acidophilus* NCFM that shared 58 % identity with *LBA649*. Interestingly, no homologue was identified in the genome of *L. helveticus* DPC4571 which shares approximately 70 % of its gene content with *L. acidophilus* NCFM. Homologues were also observed in species other than lactobacilli. These included *Streptococcus pyogenes* (~ 67 % identity), *Streptococcus agalactiae* (~ 66 % identity), *Enterococcus faecalis* (~ 65 % identity) and *Staphylococcus aureus* (~ 60 % identity).

Construction of a deletion in the *LBA649* gene

A mutant strain of *L. acidophilus* NCFM with a deletion in the *LBA649* gene was constructed using the *upp*-based counterselection system (Goh *et al.*, 2009). This system utilizes *upp* as a counterselective marker for the existing pORI-based knockout system for positive selection of double recombinants (Goh *et al.*, 2009). An in-frame deletion was constructed by amplifying approximately 780 bp of the flanking regions of the target, joining these regions using splicing by overlap extension PCR and subsequent ligation into a pORI-based counterselective

integration vector, pTRK935. In total, 1692 bp of the *LBA649* gene was deleted, leaving 84 bp (or 28 aa) in-frame in the mutant strain which was designated NCK2015 (Δ *LBA649*). This strain was compared with the parent strain NCK1909 (control for the *upp* system) in subsequent phenotypic assays.

Phenotypic analysis of the *LBA649* gene deletion strain NCK2015

Growth under different stress conditions. Although there appeared to be a slight difference in the optical density (OD_{600}) between the mutant and control over a period of 24 h growth in MRS broth (Fig. 2a), the cell numbers (c.f.u. ml^{-1}) of both strains were not significantly different over the growth period (data not shown). Subsequently, NCK2015 and NCK1909 were grown under different stress conditions to determine if the deletion of the *LBA649* gene had an effect on the ability of NCK2015 to respond to these growth conditions. The addition of 0.5 % oxgall to the MRS broth did not result in a reduction of growth of NCK2015 compared to NCK1909 (Fig. 2b). However, growth in porcine bile did result in growth inhibition of the mutant compared to the wild-type parent (NCK1909, Fig. 2e). In addition, NCK2015 demonstrated a longer lag phase and slower growth in sodium lactate (Fig. 2c), sodium acetate (Fig. 2d) and sodium chloride (Fig. 2f) compared to the parent strain NCK1909. Overall NCK2015 demonstrated a more susceptible phenotype toward

stressful environments compared to the parent strain NCK1909, indicating a role for LBA649 in stress tolerance.

Additional stress challenge assays were undertaken in the presence of the surfactants SDS and Triton X-100. Strains were grown for 24 h in MRS broth or in MRS broth containing either 0.02 % SDS or 0.5 % Triton X-100. NCK2015 demonstrated a higher susceptibility to 0.02 % SDS, whereas no difference in susceptibility was observed after exposure to 0.5 % Triton X-100 compared to the parent strain NCK1909 (Fig. 3). In fact, the mutant appeared to tolerate Triton X-100 better than the control, although this difference was not significant. Initial analyses by light microscopy revealed that both wild-type and mutant strains demonstrated different cell morphologies under the different conditions tested (data not shown). Both strains were analysed with a scanning electron microscope after growth for 24 h in MRS broth. The results showed that after growth in MRS broth the mutant NCK2015 cells were longer ($3.68 \pm 0.43 \mu\text{m}$) than the parent NCK1909 cells ($1.96 \pm 0.11 \mu\text{m}$) (Fig. 4). These results demonstrate that LBA649 plays a role in surfactant tolerance and cell morphology.

Adhesion of NCK2015 to Caco-2 cells. The ability of cultures to adhere to Caco-2 cells was measured *in vitro* using the LBA649 gene deletion strain NCK2015 compared to the parent strain NCK1909. Caco-2 cells were exposed to exponential- and stationary-phase cultures for 1 h at 37 °C to determine if growth phase affected adhesion rates. A reduction in adherence of NCK2015 (ΔLBA649) to Caco-2 cells of 50 and 45 % was observed for exponential- and stationary-phase cultures, respectively, compared to

NCK1909 (Fig. 5). Therefore, deletion of the LBA649 gene resulted in a reduced ability of the mutant strain to adhere to Caco-2 cells.

DISCUSSION

Gene replacement or insertion systems to delete or inactivate specific genes of interest (Goh *et al.*, 2009; Russell & Klaenhammer, 2001) have facilitated the confirmation of important probiotic traits of *L. acidophilus*, including bile tolerance (Pfeiler & Klaenhammer, 2009; Pfeiler *et al.*, 2007), acid stress (Azcarate-Peril *et al.*, 2004), transport and catabolism of fructooligosaccharide (Barrangou *et al.*, 2003), oxalate degradation (Azcarate-Peril *et al.*, 2006) and bacteriocin transport (Dobson *et al.*, 2007). We used the recently developed *upp*-based counterselection gene replacement system to determine the role of LBA649 in *L. acidophilus* NCFM. This system was developed to improve the pORI gene knockout system by providing a direct selection method for plasmid-free recombinants (Goh *et al.*, 2009; Russell & Klaenhammer, 2001).

An MCRA protein was first identified in *S. pyogenes* where it was suggested to play a role in the pathogenesis of streptococcal infections based on screening for antigens recognized by antibodies purified from acute rheumatic fever sera (Kil *et al.*, 1994). Although the genes encoding MCRAs contain a conserved domain and are found in many Gram-positive and Gram-negative bacteria, including probiotic bacteria, the function of these proteins is unknown in many species. Recently, MCRAs from *S. pyogenes* and *Pseudomonas* sp. were demonstrated to have

Fig. 2. Strains NCK1909 (black diamonds) and NCK2015 (grey squares) were inoculated (1 %) and grown for 24 h in MRS broth (a) and MRS broth containing 0.5 % oxgall (b), 1.8 % sodium lactate (c), 2 % sodium acetate (d), 0.2 % porcine bile (e) or 2 % NaCl (f) to produce growth curves in microtitre plates. Results shown are representative of results performed at least three times for each growth condition.

Fig. 3. Survival of strains NCK1909 (black bars) and NCK2015 (grey bars) in MRS broth supplemented with 0.02 % SDS or 0.5 % Triton X-100 after 24 h at 37 °C. Percentage survival is defined as (c.f.u. ml⁻¹ at *t*=24 h/c.f.u. ml⁻¹ at *t*=0 h)×100. Results shown are the means ±SD of four independent assays. A Student's *t*-test indicated that the results are significantly different (*) at a value of *P*<0.05 for survival of the mutant compared to the control in 0.02 % SDS, but not for the mutant compared to the control in 0.5 % Triton X-100 (*P*>0.05).

Fig. 5. Percentage adhesion of exponential-phase (grey bars) and stationary-phase (black bars) cells of strain NCK1909 (control) and the mutant strain NCK2015 to Caco-2 cells after 1 h incubation at 37 °C. Results are the means ±SD of three independent assays. A Student's *t*-test indicated that the results are significantly different (*) at a value of *P*<0.05 for percentage adhesion of exponential- and stationary-phase cells of the mutant compared to control cells.

hydratase activity (Bever *et al.*, 2009; Volkov *et al.*, 2010). In addition, the MCRA from *S. pyogenes* was shown to contribute to blood survival and adherence to keratinocytes (Volkov *et al.*, 2010). Analysis of LBA649 against proteins in the non-redundant database revealed the highest level of homology to a protein from *L. acidophilus*

annotated as a linoleate isomerase (GenBank accession number ABB43157). Currently, there is no additional information in the published literature with regard to the origin of this strain or function of the protein. Previous work demonstrated that *L. acidophilus* NCFM and the neotype strain *L. acidophilus* ATCC 4356 did not produce conjugated linoleic acid from linoleic acid (Coakley *et al.*, 2003; Jenkins & Courtney, 2003). However, it remains to be determined if the purified protein product encoded by the LBA649 gene exhibits hydratase or linoleic activity. Interestingly, the gene order surrounding LBA649 was conserved in other lactobacilli with sequenced genomes, such as the stomach isolate *L. ultunensis* DSM 16047 and to a lesser extent *L. gasseri* ATCC 33323. Comparison with the region in the vicinity of the MCRA orthologue in *Streptococcus pyogenes* and *Staphylococcus aureus* demonstrated no conserved gene order compared to the corresponding region in *L. acidophilus*. In addition, *in silico* analysis identified a second gene (LBA555) in the genome of *L. acidophilus* NCFM annotated as an MCRA. Strains containing a single mutation of this gene and a double mutation in both genes were constructed (unpublished data). Preliminary analyses indicated that the single mutation in LBA555 did not show the same phenotype as NCK2015. Additionally, the strain containing the double deletion demonstrated the same phenotype as the single deletion in the LBA649 gene, indicating that deleting LBA555 did not contribute to the phenotype observed for LBA649.

Fig. 4. Scanning electron micrographs of the control NCK1909 (a) and the mutant NCK2015 (b) strains after growth for 24 h in MRS broth. Bars, 1 μm.

Features of probiotic bacteria include their ability to adhere to epithelial cells and to resist stresses such as bile and acid stress. Growth of the mutant strain (ΔLBA649) in MRS broth containing sodium lactate resulted in a lag phase for the mutant of 12 h compared to 8 h for the parent strain. An increased lag phase and inhibition of growth for the

mutant was also observed during growth in sodium acetate. The incorporation of NaCl to the medium exhibited a greater inhibitory effect on the mutant strain compared to the control strain. Interestingly, growth of the control and mutant strain was comparable in oxgall; however, there was greater inhibition of the mutant when grown in porcine bile compared to the parent strain. Although oxgall is commonly used to study the tolerance of strains to bile, porcine bile is more similar to human bile and more inhibitory than oxgall (Begley *et al.*, 2005). NCK2015 was also more susceptible than NCK1909 to SDS. These data indicate that LBA649 contributes to stress tolerance under these growth conditions. Perhaps by deleting the LBA649 gene, cell morphology and surface structure were altered, thereby affecting the ability of the mutant cells to tolerate these substances.

Intestinal epithelial cells are the first cells to interact with resident or transient bacteria of the intestine, wherein probiotic bacteria can have a positive effect on epithelial tight junctions, and can also initiate and regulate the immune response at the mucosa (O'Flaherty & Klaenhammer, 2010). The adhesion ability of probiotic bacteria and competitive exclusion of pathogens has been studied *in vitro* using cell lines such as HT-29 and Caco-2 (Buck *et al.*, 2005; Gopal *et al.*, 2001; Lin *et al.*, 2008; van Pijkeren *et al.*, 2006). Previous work with *L. acidophilus* NCFM using an integration system to inactivate and knock out specific cell surface factors demonstrated the involvement of these factors in adhesion to Caco-2 cells (Buck *et al.*, 2005). Additional work demonstrated the involvement of LuxS in adhesion to Caco-2 cells (Buck *et al.*, 2009) and deletion of the gene encoding sortase from *Lactobacillus salivarius* resulted in reduced adherence to human epithelial cell lines (van Pijkeren *et al.*, 2006). These studies demonstrate that a bacterium may encode numerous cell-surface factors that are involved in adherence. We measured the ability of the mutant strain to adhere to Caco-2 cells after exponential- and stationary-phase growth. Both growth phases demonstrated a reduction in the capability of the mutant strain to adhere to the human intestinal epithelial cells, demonstrating the involvement of LBA649 in adherence.

The availability of genome sequences and tools for deleting or inactivating genes of interest for various probiotic species has provided an in-depth view of the genetic content that programmes the organisms' ability to compete, survive and persist within the gastrointestinal tract. However, a future challenge is to determine the contribution of genes that may not have an obvious function that is apparent from their annotation or placement in an operon. Nevertheless these genes may have an important role to play in probiotic functionality. The LBA649 gene is one example, and this study has demonstrated a function for the protein it encodes in stress tolerance and in adherence to Caco-2 cells, both of which are important probiotic bacteria attributes.

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