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Construction of vectors for inducible and constitutive gene expression in *Lactobacillus*

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Summary

Microarray analysis of the genome of *Lactobacillus acidophilus* identified a number of operons that were differentially expressed in response to carbohydrate source or constitutively expressed regardless of carbohydrate source. These included operons implicated in the transport and catabolism of fructooligosaccharides (FOS), lactose (*lac*), trehalose (*tre*) and genes directing glycolysis. Analysis of these operons identified a number of putative promoter and repressor elements, which were used to construct a series of expression vectors for use in lactobacilli, based on the broad host range pWV01 replicon. A β-glucuronidase (GusA3) reporter gene was cloned into each vector to characterize expression from each promoter. GUS reporter assays showed FOS, *lac* and *tre* based vectors to be highly inducible by their specific carbohydrate and repressed by glucose. Additionally, a construct based on the phosphoglycerate mutase (*pgm*) promoter was constitutively highly expressed. To demonstrate the potential utility of these vectors, we constructed a plasmid for the overexpression of the oxalate degradation pathway (Frc and Oxc) of *L. acidophilus* NCFM. This construct was able to improve oxalate degradation by *L. gasseri* ATCC 33323 and compliment a *L. acidophilus* oxalate-deficient mutant. Development of these expression vectors could support several novel applications, including the expression of enzymes, proteins, vaccines and biotherapeutics by intestinal lactobacilli.

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Introduction

Lactobacilli are members of the lactic acid bacteria, a functional group related by formation of lactic acid as the primary product of carbohydrate metabolism. Lactobacilli have long been considered beneficial, occupying important niches in the gastrointestinal (GI) tracts of humans and animals. Select lactobacilli are increasingly recognized as modulators of health, gaining interest as microbes used as health-promoting functional food ingredients, and as delivery vectors for vaccines and biotherapeutics (Wells *et al*., 1996).

There continues to be great interest in the development of genetic tools for production of proteins and enzymes from lactic acid bacteria. Several gene expression systems for lactobaclli have been developed. The widely used nisin-controlled expression (NICE) system, originally developed for use in *Lactococcus lactis* (de Ruyter *et al*., 1996), has been adapted for use in lactobacilli (Kleerebezem *et al*., 1997; Wu *et al*., 2006). Expression systems based on control by other bacteriocins (Axelsson *et al*., 2003; Mathiesen *et al*., 2004) and lactose (Gosalbes *et al*., 2001) are also available.

Lactobacillus acidophilus NCFM is a probiotic culture widely used in nutritional supplements, dairy products and infant formulas. The availability of the *L. acidophilus* genome sequence (Altermann *et al*., 2005), gene expression profiling (Azcarate-Peril *et al*., 2005; 2006; Barrangou *et al*., 2006) and functional genomic studies (Russell and Klaenhammer, 2001a; Barrangou *et al*., 2003; Duong *et al*., 2006) has provided considerable insight into the physiology of this organism and established a technical basis that can be used to improve both the fermentation and probiotic functionalities of this organism.

In this study, we exploit the genome sequence, gene expression profiling and functional genomic data to construct a series of expression vectors and analyse their properties using a β-glucuronidase (GusA3) reporter protein. Additionally, one vector encoding a strong constitutive promoter was employed for overexpression of the

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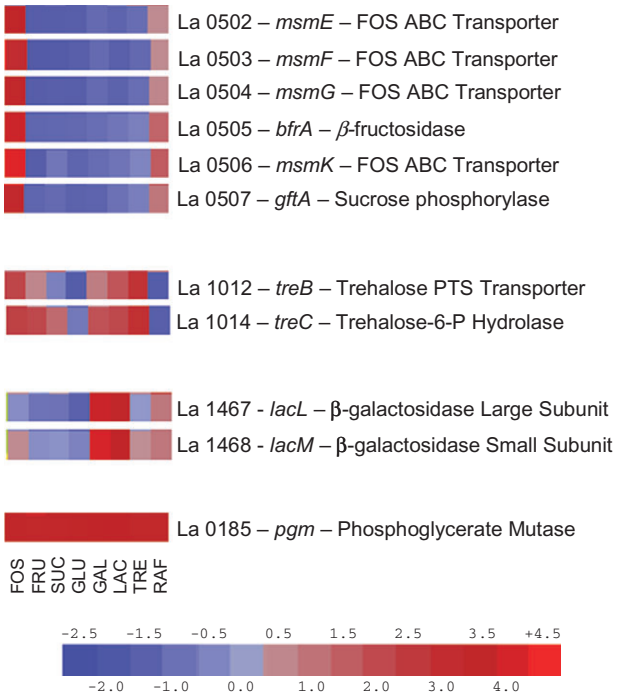

Fig. 1. Microarray expression data for select genes and operons. Gene expression of FOS, *lac* and *tre* operons and the *pgm* gene in *L. acidophilus* grown on fructooligsaccharides (FOS), fructose (FRU), sucrose (SUC), glucose (GLU), galactose (GAL), lactose (LAC), trehalose (TRE) and raffinose (RAF) is shown colormetrically. Scale represents least squares means of overall gene expression level.

L. acidophilus oxalate-degradation operon and complementation of a deletion mutation therein (Azcarate-Peril *et al.*, 2005).

Results

Promoter selection

Previous global gene expression analysis found a number of genes involved in carbohydrate transport and metabolism to be differentially expressed during growth on various carbohydrates (Barrangou *et al.*, 2006). Also, a number of genes were identified as being highly constitutively expressed (Fig. 1). Putative promoters (Fig. 2) from the FOS (P_{FOS}), *lac* (P_{lac}) and *tre* (P_{tre}) operons (Fig. 3) were selected for use in the construction of carbohydrate-inducible vectors, while the promoter for *pgm* (P_{pgm}) was selected for use in the construction of a constitutive expression vector.

Terminator selection

Lactobacillus acidophilus NCFM sequences that could potentially function as Rho-independent terminators were identified by dyad symmetry analysis using Clone Manager (SciEd, Durham, NC, USA) and TransTerm (Ermolaeva *et al.*, 2000). Two putative terminators, Term908 and Term667, were selected for use in this series of expression vectors (Table 1). Terminators were selected based upon the following criteria: low Gibbs free energy ($\Delta°G$) (Term908: $\Delta°G = -24.4$ kcal mol^{-1}; Term667: $\Delta°G = -23.5$ kcal mol^{-1}), location within a 'head-to-tail' intergenic region (Fig. 3) and, TransTerm confidence score (100 for both). Additionally, both terminators were predicted to function as bidirectional terminators by TransTerm. Transcription arrays from Barrangou and colleagues (2006) also showed cessation of expression of downstream genes.

Plasmid construction

The vectors constructed in this study are shown in Fig. 4. Terminators were cloned into pTRK846, sequentially. Transformants were screened and selected in order maintain the native 'head-to-tail' orientation from *L. acidophilus* to prevent transcription into the expression cassette. The resulting construct, pTRK847, was used as the base for this series of expression vectors into which regulatory elements for the FOS (pTRK848), *lac* (pTRK849), *tre* (pTRK850) operons and *pgm* (pTRK882) were cloned. Plasmid construction was confirmed by sequencing. The *Lactobacillus gusA3* gene from pTRK782 (Callanan *et al.*, 2007) was directionally cloned into each vector to create plasmids pTRK888 (pFOS), pTRK889 (pLAC), pTRK890 (pTRE) and pTRK892 (pPGM) for expression analysis (Table 2).

GUS activity

The GUS assays were performed using *L. acidophilus* NCFM and *Lactobacillus gasseri* ATCC 33323 transformants that carried the GUS reporter constructs. A time-course experiment was performed using cultures of *L. acidophilus* NCK 1825, harbouring the FOS-inducible GUS reporter construct, pTRK888. GUS activity of cell-free extracts (CFEs) made from 10 ml aliquots of culture taken at 0, 1, 2 and 3 h post induction was determined (Fig. 5). Cultures induced with FOS had a 100-fold

Table 1. Transcriptional terminators.

Terminator	$\Delta°G$ (kcal mol^{-1})	Sequence
Term 908	–24.4	UAAUCUGAAGAAAAAGGAGGCUAG $^{U}_{A}$ AUUAGAUUUCUUUUUCCUCCGAUC $^{A}_{U}$
Term 667	–23.5	AAAUAACAAAAAGAGUAUGAG $^{UU}_{U}$ UUUAUUGUUUUUCUCAUACUC $^{GU}_{U}$

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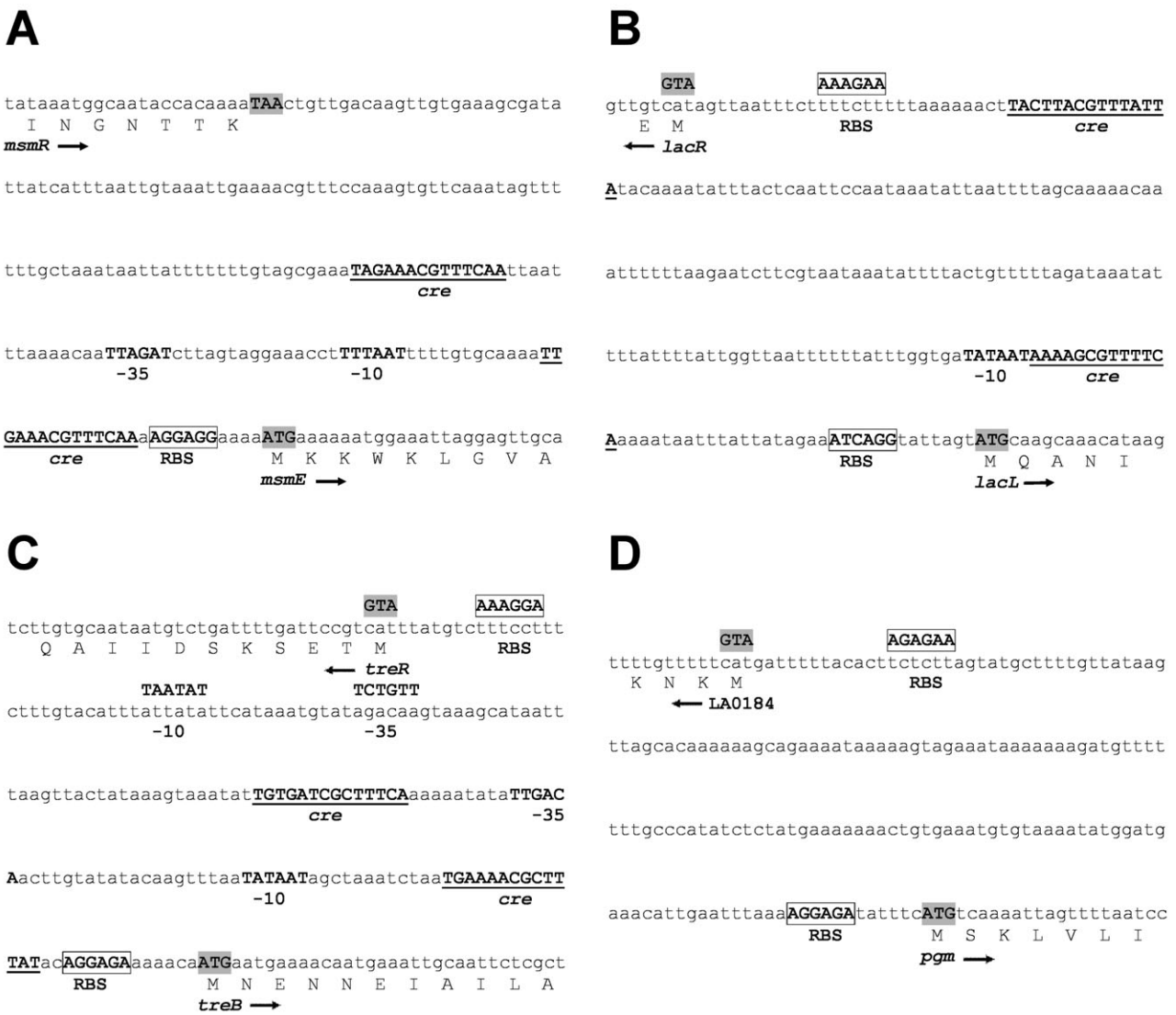

Fig. 2. Nucleotide sequence of select intergenic regions. Native promoter regions for (A) FOS, (B) *lac* and (C) *tre* operons and (D) *pgm*. The consensus catabolite response element (*cre*) sequence WTGNAANCGNWNNCW (Barrangou *et al*., 2003) was used to identify *cre*-like sequences in the promoter regions. Putative –35 and –10 regions are in bold; putative *cre*-like sequences are underlined; putative ribosome binding sites (RBS) are boxed; and putative translation start sites are in shaded text.

greater GUS activity at 1, 2 and 3 h post induction as compared with cultures exposed to a non-inducing carbohydrate, fructose, and a repressive carbohydrate, glucose. No GUS activity was detected at 0 h post induction, indicating that GUS expression was undetectable in MRS medium. Since no significant increase in specific activity was detected at 2 and 3 h post induction by FOS, GUS activity for the remaining carbohydrate inducible and constitutive expressing constructs was assayed at 1 h post induction.

The GUS activity of cultures of *L. acidophilus* and *L. gasseri* harbouring pTRK888, pTRK889 and pTRK890 exposed to an inducing carbohydrate, fructose and glucose was determined at 1 h post induction. The GUS activity was greatest in each strain exposed to its specific inducing carbohydrate. In each case, a detectable level of basal expression from the inducible promoters was observed in cells exposed to glucose (lowest) or fructose (intermediate) (Fig. 6). In *L. acidophilus,* induction of GUS activity by the inducing carbohydrates was 18-fold higher in pTRK888, 50-fold higher in pTRK889, and 14-fold higher in pTRK890 as compared with glucose. In *L. gasseri* GUS activity was induced 16-fold in pTRK888, fourfold in pTRK889 and fivefold in pTRK890. Additionally, when specifically induced, GUS activity was found to be at least 10-fold higher in CFEs from *L. gasseri* than in CFEs from *L. acidophilus*.

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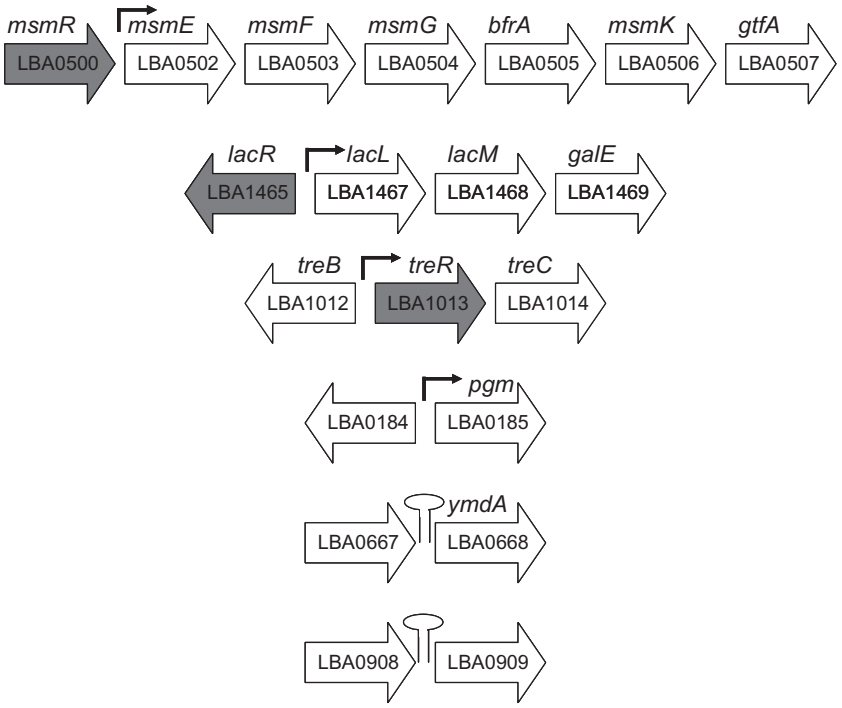

Fig. 3. Layouts of selected operons and genes. Open reading frames (ORFs) are represented by arrows with ORF number inside the arrow and gene names above. Transcriptional regulators are shown in grey; promoters are shown as black arrows; and Rho-independent terminators shown as stem loops. ORF representations are not shown to scale.

The GUS activity of *Lactobacillus* cultures harbouring pTRK892 (P_{pgm}) when exposed to different carbohydrates was also determined (Fig. 7). The GUS activity was found to be highly expressed across all conditions in both organisms with levels of expression approximately 10-fold over the levels observed with the induced constructs. There was no significant difference in GUS activity between carbohydrates. GUS activity was found to be threefold to 16-fold higher in CFEs from *L. gasseri* than in CFEs from *L. acidophilus*.

Analysis of oxalate degradation in recombinant Lactobacillus

In order to determine the effect of overexpressing the *L. acidophilus* NCFM oxalate operon on oxalate degradation by lactobacilli, pTRK928 (Fig. 8) was transformed into *L. acidophilus* NCFM (NCK 56), an *L. acidophilus frc* deletion mutant (NCK 1728), *L. gasseri* ATCC 33323 and *L. gasseri* ADH. The empty vector, pTRK882, was transformed into these strains to serve as controls. The ability of *L. acidophilus* (Fig. 9A) and *L. gasseri* (Fig. 9B) cultures to degrade oxalate over 96-hours was determined. Oxalate degradation in the *L. acidophilus* strains was 5–52% over the 96-hour time-course. The *frc* deletion mutant strain harbouring the control plasmid (NCK 1899) degraded only 5% of the oxalate, while the *frc* deletion harbouring pTRK928 (NCK 1897) degraded 48%. Additionally, there was no significant difference in oxalate degradation by *L. acidophilus* NCFM harbouring pTRK928 or the empty vector. While expression of the oxalate operon using pTRK928 showed no significant improvement in the oxalate degradation ability of *L. acidophilus* NCFM, pTRK928 was able to complement the deletion mutation, improving oxalate degradation in the *frc* mutant from 5% to 48%. Oxalate degradation in the *L. gasseri* strains was 20–45% over 96 h with the *L. gasseri* ATCC 33323-derived strains degrading 10–25% greater amounts of oxalate than the *L. gasseri* ADH-derived strains. No significant difference in oxalate degradation was measured between the *L. gasseri* ADH-derived strains, indicating that pTRK928 was unable to confer improved oxalate degrading ability on this particular strain. However, *L. gasseri* ATCC 33323 harbouring pTRK928 (NCK 1969) degraded 15% more oxalate than its control (NCK 1970), indicating that pTRK928 was able to improve oxalate degrading ability of the parent strain.

Discussion

Gene expression analysis in *L. acidophilus* using microarrays identified a number of operons related to carbohydrate metabolism whose expression was tightly regulated by carbohydrates (Barrangou *et al*., 2006). Specifically, genes within these operons were found to be highly induced when the organism was grown on a specific carbohydrate and to be very highly repressed when grown on glucose. Based on these data and previous functional characterization using gene insertion knockouts, the FOS

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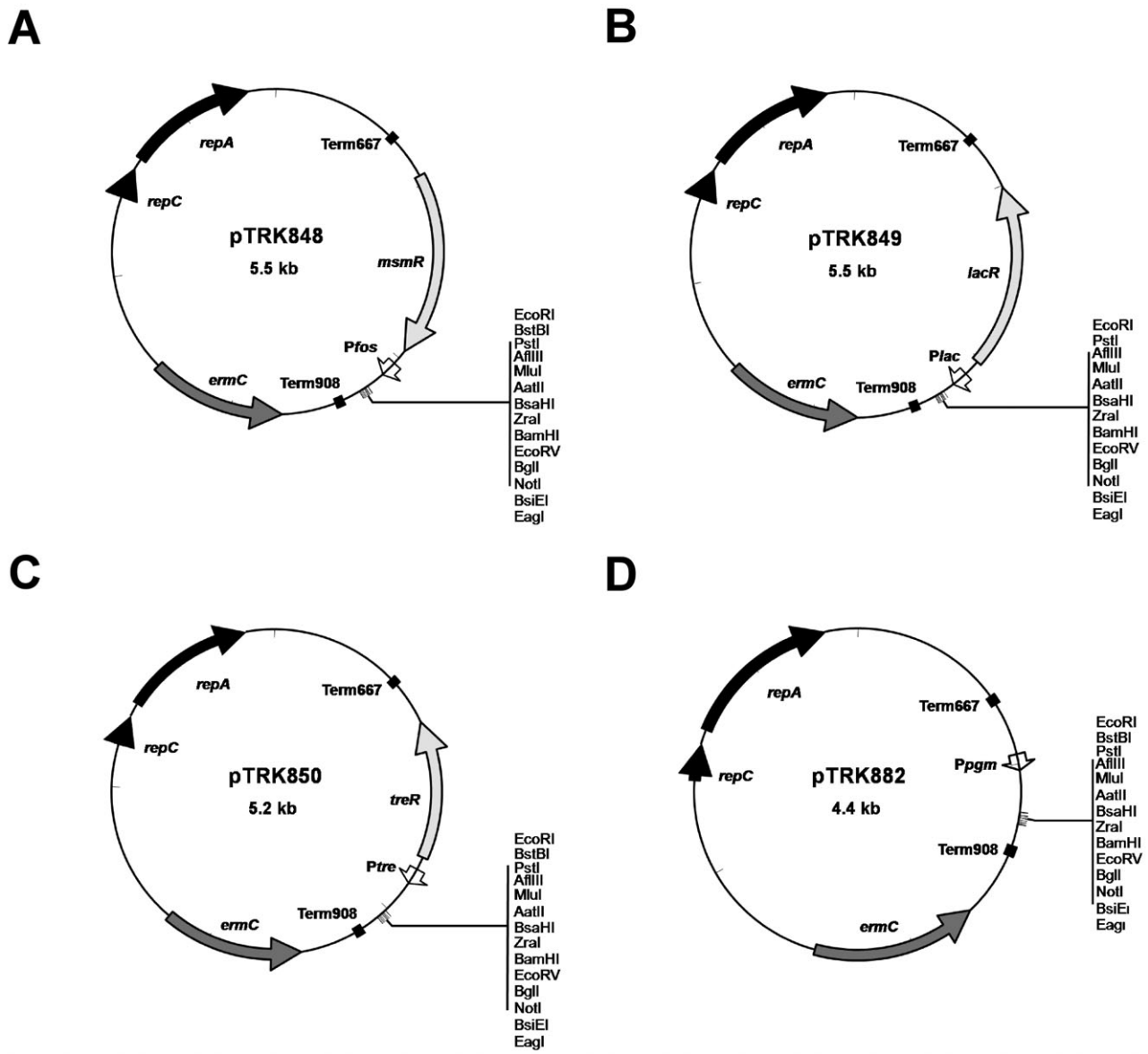

Fig. 4. Construction of expression vectors. Restriction maps for expression vectors. Details of construction are given in the text. Black arrows, replication determinants; dark grey arrows, antibiotic resistance markers (*ermC*: erythromycin resistance maker); black boxes, transcriptional terminators; light grey arrows, transcriptional regulators (*msmR*: FOS repressor; *treR*: trehalose repressor; *lacR*: lactose repressor); white arrows, promoters (P_{fos}: FOS promoter; P_{lac}: *lac* promoter; P_{tre}: *tre* promoter; P_{pgm}: *pgm* promoter). All restriction sites shown are unique.

(Barrangou *et al*., 2003), *lac* (Russell and Klaenhammer, 2001a) and *tre* (Duong *et al*., 2006) operons were selected to serve as the basis for our carbohydrate-inducible vectors.

β-Glucuronidase reporter assays show the inducible vectors to be inducible and regulated by carbohydrates in both *L. acidophilus* and *L. gasseri*. In all strains, the inducing carbohydrates elicited the highest GUS activity, while glucose repressed GUS activity by a factor of 4 or more. GUS activity of pTRK890 (P_{tre}) showed the least difference in activity between trehalose and glucose in both organisms. This was in agreement with the microarray expression data in *L. acidophilus* and is most likely due to trehalose present in yeast extract used to prepare media. GUS activity of *Lactobacillus* cultures harbouring pTRK892 (P_{pgm}) was not found to be significantly different when exposed to MRS or different carbohydrates but, in all cases, was constitutively highly expressed at over 10-fold over levels observed with inducible vectors. The results showed that the expression systems maintain regulation of expression similar to the native genes positioned in the *L. acidophilus* genome. Modified *Lactobacillus* GusA3

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Table 2. Bacterial strains and plasmids.

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
L. acidophilus		
NCK 56	NCFM, human intestinal isolate	Barefoot and Klaenhammer (1983)
NCK 1825	NCFM w/ pTRK888	This study
NCK 1826	NCFM w/ pTRK889	This study
NCK 1827	NCFM w/ pTRK890	This study
NCK 1829	NCFM w/ pTRK892	This study
NCK 1728	NCFM, Δ*frc* (Lba0395)	Azcarate-Peril *et al.* (2006)
NCK 1889	NCFM w/ pTRK928	This study
NCK 1895	NCFM w/ pTRK882	This study
NCK 1897	NCK 1728 w/ pTRK928	This study
NCK 1899	NCK 1728 w/ pTRK882	This study
L. gasseri		
ATCC 33323	Neotype	American Type Culture Collection
NCK 1935	ATCC 33323 w/ pTRK888	This study
NCK 1936	ATCC 33323 w/ pTRK889	This study
NCK 1937	ATCC 33323 w/ pTRK890	This study
NCK 1938	ATCC 33323 w/ pTRK892	This study
NCK 99	ADH, Human intestinal isolate	Kleeman and Klaenhammer (1982)
NCK 1967	ADH w/ pTRK928	This study
NCK 1968	ADH w/ pTRK882	This study
NCK 1969	ATCC 33323 w/ pTRK928	This study
NCK 1970	ATCC 33323 w/ pTRK882	This study
E. coli		
MC 1061	Str^r, *E. coli* transformation host	Casadaban and Cohen (1980)
NCK 1751	MC 1061 w/ pTRK846	This study
NCK 1752	MC 1061 w/ pTRK847	This study
NCK 1753	MC 1061 w/ pTRK848	This study
NCK 1754	MC 1061 w/ pTRK849	This study
NCK 1755	MC 1061 w/ pTRK850	This study
NCK 1814	MC 1061 w/ pTRK882	This study
Plasmids		
pGK12	ori (pWV01), Em^r, Cm^r, Gram-positive shuttle vector	Kok *et al.* (1984)
pORI28	ori (pWV01), Em^r, source of MCS	Law *et al.* (1995)
pTRK782	Source of *gus*A3 reporter gene	Callanan *et al.* (2007)
pTRK846	Em^r, Δ*cat* derivative of pGK12 with MCS from pORI28	This study
pTRK847	Em^r, pTRK846 with Term 908 and Term 667	This study
pTRK848	1.4-kb *msm*R/P_{FOS} PCR fragment cloned into pTRK847	This study
pTRK849	1.4-kb *lacR*/P_{lac} PCR fragment cloned into pTRK847	This study
pTRK850	1.4-kb *treR*/P_{tre} PCR fragment cloned into pTRK847	This study
pTRK882	350-bp P_{pgm} PCR fragment cloned into pTRK847	This study
pTRK888	*gus*A3 cloned into pTRK848	This study
pTRK889	*gus*A3 cloned into pTRK849	This study
pTRK890	*gus*A3 cloned into pTRK850	This study
pTRK892	*gus*A3 cloned into pTRK882	This study
pTRK928	7.6 kb, pTRK882 with 3.1 kb PCR product from primers OXF/OXR	This study

enzyme was previously shown to offer superior performance and reproducibility in acidifying conditions over native *Lactobacillus* GusA enzyme (Callanan *et al.*, 2007).

Additional benefits may be gained by using these specific carbohydrates. Fructooligosaccharides are prebiotic compounds thought to promote the growth of probiotic microorganisms in the GI tract (Gibson and Roberfroid, 1995). Fructooligosaccharides remain undigested by the GI tract and are available to the lactobacilli in the lower GI tract. By using a FOS-inducible expression vector, it may be possible to target the expression of desired proteins in the lower GI tract, where the undigested FOS are more prevalent. This may be useful for the use of lactobacilli as live vaccine or biotherapeutic delivery vectors. Trehalose is a glucose disaccharide commonly used as a cryoprotectant. It has been shown to confer protection from freezing, heat and desiccation stress. This conferred protection may benefit the end-user of a trehalose-inducible expression system. Lactose is a very inexpensive and readily available carbohydrate and the primary carbohydrate source available in milk, potentially facilitating the use of lactose-inducible vectors in dairy applications.

Previous efforts at developing a FOS-inducible expression vector in our group found expression from a pGK12-derived vector using only the FOS promoter to be very leaky (Miller *et al.*, 2004). This was possibly due to repressor titration (Williams *et al.*, 1998) or transcriptional readthrough from promoters outside the expression cas-

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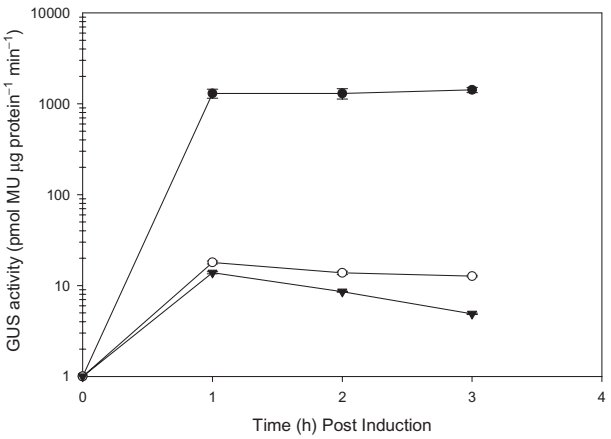

Fig. 5. Time-course of induction. CFEs of *L. acidophilus* harbouring pTRK888 (P_{FOS}) were assayed for GUS activity at 0, 1, 2 and 3 h post induction. Cultures were induced in (●) FOS, (o) fructose or (▼) glucose. Error bars represent the standard error of the means (SEM) for three independent experiments.

sette (Gibson *et al*., 1987). In order to avoid this, the respective repressors from each operon and Rho-independent terminators were included in the vectors constructed for this study.

Gosalbes and colleagues (2001) previously described an expression vector based on the lactose operon in *Lactobacillus casei* ATCC 393. This vector was based on pIAβ5, containing a p15A replicon from pACYC184, and functioned only in *L. casei*. The expression systems described herein are based on the pGK12 shuttle replicon and contain determinants, which allow replication in both *Escherichia coli* and most lactic acid bacteria (Kok *et al*., 1984). This makes it possible to build constructs in *E. coli* for eventual transformation into lactobacilli and, likely, other lactic acid bacteria. While we have only tested this system in *L. acidophilus* and *L. gasseri*, we expect that this series of vectors will be useful in the wide host range available to pGK12.

Absorption of dietary oxalate in the colon contributes to hyperoxaluria (Balaji and Menon, 1997) and increases the risk of urinary calcium oxalate stone formation. Because humans lack enzymes for the breakdown of oxalate, degradation by intestinal bacteria is important to maintaining the oxalate homeostasis (Stewart *et al*., 2004). The use of oxalate degrading lactic acid bacteria has been demonstrated as a potential biotherapeutic route for the treatment of oxalate-related disorders in humans (Campieri *et al*., 2001; Lieske *et al*., 2005). *Lactobacillus acidophilus* and *L. gasseri* have been previously identified as oxalate degrading probiotic lactobacilli (Azcarate-Peril *et al*., 2006; Lewanika *et al*., 2007). To demonstrate the utility of constructs developed in this study, we investigated the possibility of improving the oxalate degrading activity of these organisms by overexpression of the *L. acidophilus* oxalate gene cluster. In this study the promoter for the constitutively highly expressed gene *pgm* was used in pTRK928 to drive expression of the oxalate operon. Plasmid pTRK928 was able significantly improve the oxalate degradation activity of *L. gasseri* ATCC 33323, suggesting that this strategy may be effective in other strains or may be used to add oxalate degrading activity to organisms unable to degrade oxalate. Additionally, the overexpression of *oxc* and *frc* using pTRK928 was able to return the oxalate degradation activity of the *L. acidophilus frc* deletion mutant (NCK 1724) to the level of the wild-type parent strain. This illustrates the potential use of the pTRK882 expression system in complementation studies.

In this work, a series of expression vectors designed for use in lactobacilli are described. The GUS reporter

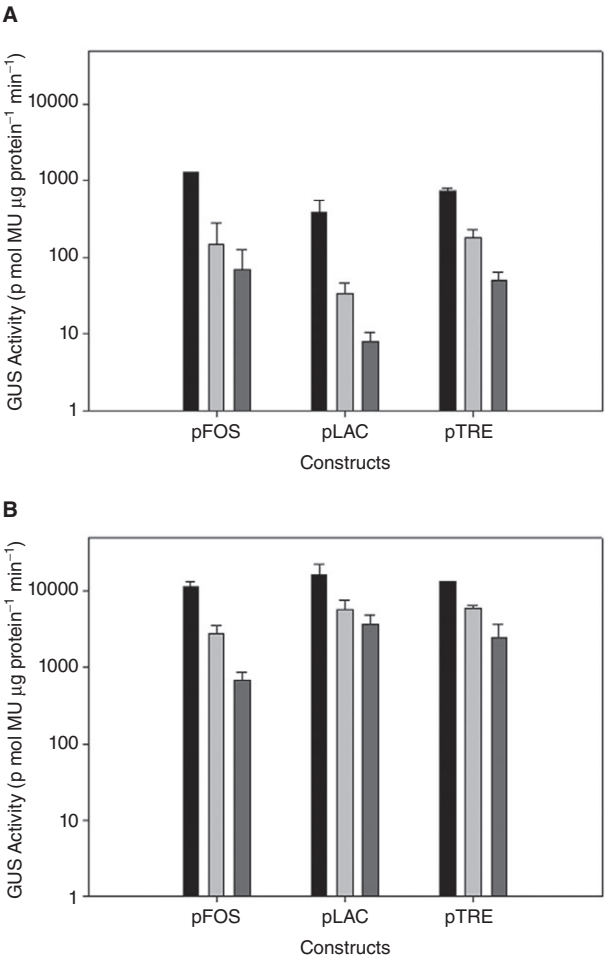

Fig. 6. β -Glucurondiase activity of inducible vectors. GUS activity of (A) *L. acidophilus* and (B) *L. gasseri* harbouring pTRK888 (pFOS), pTRK889 (pLAC) and pTRK890 (pTRE) in inducing carbohydrate (black), fructose (light grey) and glucose (dark grey) at 1 h post induction. Inducing carbohydrates were as follows: pTRK888, FOS; pTRK889, lactose; pTRK890, trehalose. Error bars represent the SEM for three independent experiments.

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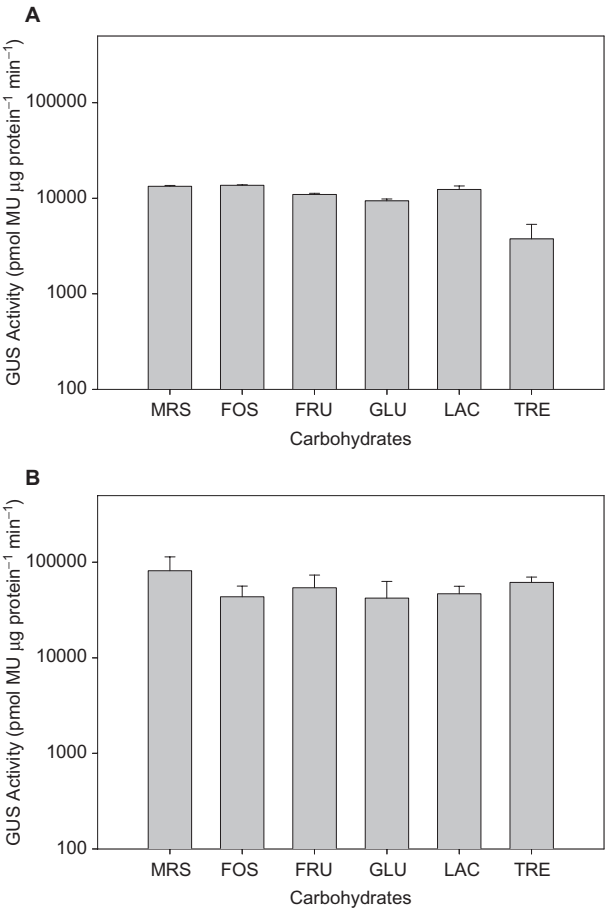

Fig. 7. β -Glucurondiase activity of constitutive vector. GUS activity of (A) *L. acidophilus* and (B) *L. gasseri* harbouring pTRK892 at 1 h post induction. Cultures were incubated in SSM + 1% carbohydrate or MRS. Error bars represent the SEM for three independent experiments.

gene results showed the carbohydrate-inducible expression systems to be highly inducible by their respective carbohydrates, while being significantly repressed by glucose in both *L. acidophilus* and *L. gasseri*. Additionally, the constitutive expression vector was found to produce relatively high amounts of GUS activity across all conditions. Although only tested in *L. acidophilus* and *L. gasseri*, we expect these vectors to have the same broad host range as pGK12. These expression vectors provide alternative and useful tools for overexpression of desired proteins and enzymes in lactobacilli and potentially other members of the lactic acid bacteria. Additionally, we have shown the potential of these vectors for use in genetic complementation studies and also for expression of biotherapeutic proteins (Mohamadzadeh *et al*., 2009). Additional work is now in progress to exploit these new vectors for improvement of probiotic functionalities and *in vivo* applications.

Experimental procedures

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 2. Liquid cultures of *E. coli* were propagated at 37°C in Luria–Bertani broth (Difco, Detroit, MI, USA) with shaking, while *Lactobacillus* cultures were propagated statically at 37°C in deMan, Rogosa and Sharpe (MRS) broth (Difco). Solid media was prepared with the addition of 1.5% agar (%w/v) (Difco). When appropriate, erythromycin (Fisher Scientific, Fair Lawn, NJ, USA) was added as follows: 150 μg ml^{-1} for *E. coli* and 5 μg ml^{-1} for *Lactobacillus*.

For induction experiments, *Lactobacillus* cultures were grown in MRS, harvested and resuspended in semi-synthetic medium (SSM) (Barrangou *et al*., 2003) supplemented with 1% (w/v) of the appropriate carbohydrate. The carbohydrates added to the SSM include glucose (Fisher), fructose (Sigma,

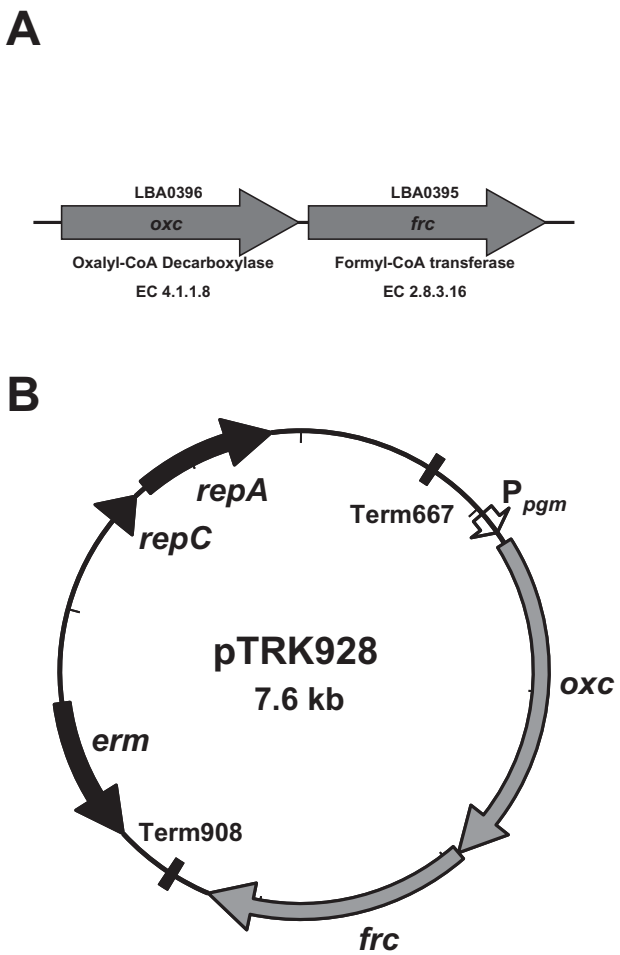

Fig. 8. Schematic of oxalate operon and plasmid.
A. *oxc* and *frc* from *Lactobacillus acidophilus* NCFM.
B. Oxalate operon expression construct, pTRK928. Promoter, $P_{pgm,}$ is shown in white; *oxc* and *frc* are shown in light grey; erythromycin resistance marker, *erm*, is shown in dark grey; replication determinants are shown in black; terminators are shown as black boxes.

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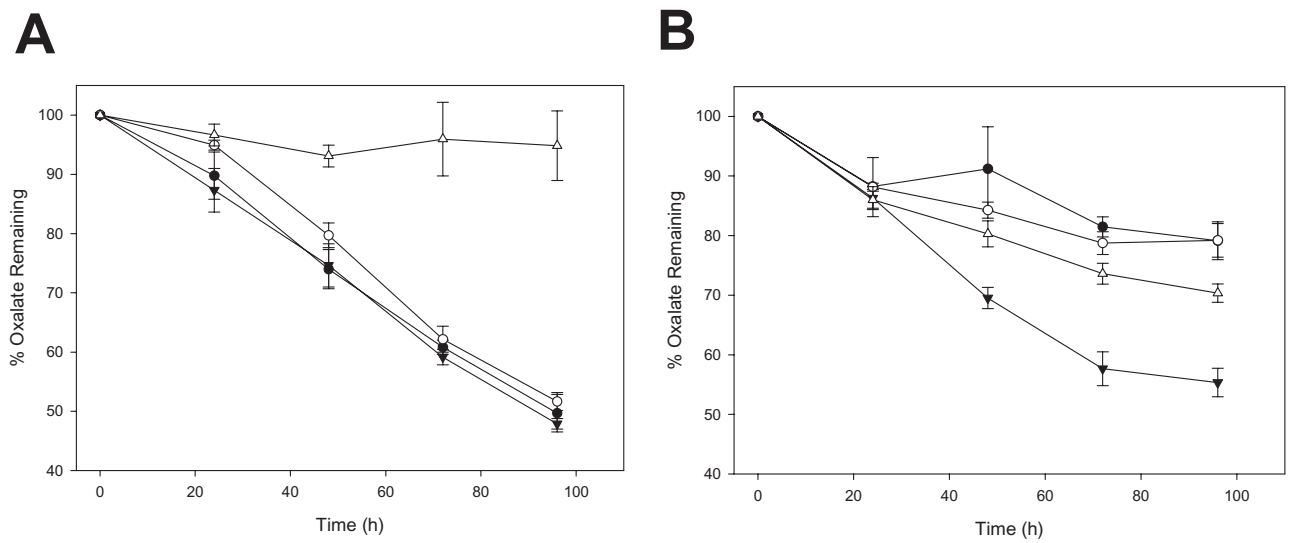

Fig. 9. Oxalate degradation by *Lactobacillus* cultures. Cultures were consecutively transferred in MRS containing 0.05% oxalate then transferred to MRS containing 0.1% oxalate. Cultures were assayed for oxalate at 0, 24, 48, 72 and 96 h after the final transfer.
A. Oxalate degrading activity of *L. acidophilus* cultures: (●) *L. acidophilus* NCFM + pTRK928 (NCK 1889), (○) *L. acidophilus* NCFM + pTRK882 (NCK 1895), (▼) *L. acidophilus frc* deletion + pTRK928 (NCK 1897), (△) *L. acidophilus frc* deletion + pTRK882 (NCK 1899).
B. Oxalate degrading activity of *L. gasseri* cultures: (●) *L. gasseri* ADH + pTRK928 (NCK 1967), (○) *L. gasseri* ADH + pTRK882 (NCK 1968), (▼) *L. gasseri* ATCC 33323 + pTRK928 (NCK 1969), (△) *L. gasseri* ATCC 33323 + pTRK882 (NCK 1970). Error bars are standard deviations of means from triplicate measurements of three independent experiments.

St Louis, MO, USA), Raftilose P95 (FOS) (Orafti, Tienen, Belgium), lactose (Sigma) and trehalose (Sigma).

DNA isolation and transformation

Lactobacillus acidophilus genomic DNA was isolated according to the method of Walker and Klaenhammer (1994). *Escherichia coli* plasmid DNA was isolated using the QIAprep Spin Miniprep (Qiagen, Valencia, CA, USA) kit. All DNA manipulations were performed according to standard procedures (Ausubel *et al*., 2001). Electrocompetent *E. coli* MC-1061 cells were prepared and transformed according to standard protocols (Ausubel *et al*., 2001). Electrocompetent *Lactobacillus* cells were prepared as described by Walker *et al*. (Luchansky *et al*., 1991; Walker *et al*., 1996). Electrotransformation was performed using a Gene-Pulser (Bio-Rad, Hercules, CA, USA).

Construction of expression vectors

Plasmids used or constructed appear in Table 2. Plasmids were constructed using standard molecular cloning techniques (Ausubel *et al*., 2001). Restriction enzymes, T4 Ligase, *taq* DNA polymerase and expand high fidelity polymerase chain reaction (PCR) system polymerase were purchased from Roche Molecular Biochemicals (Indianapolis, IN, USA). DNA fragments were purified from agarose gels using the Zymoclean Gel DNA Recovery Kit (Zymo Research, Orange, CA, USA). All PCRs were performed according to the manufacturer's instructions. PCR primers were designed using Clone Manager 9 (SciEd) and synthesized by Integrated DNA Technologies, Incorporated (Coralville, IA, USA) with restriction sites designed at the 5′ end of the primers to facilitate cloning when appropriate (Table 3). PCR products were purified using the QIAquick PCR purification kit (Qiagen). DNA sequencing was performed by Davis Sequencing (Davis, CA, USA).

Plasmid pTRK846 was derived from pGK12 (Kok *et al*., 1984) by ligating an inverse PCR product lacking the chloramphenicol resistance (Cm^r) marker to the multiple cloning site (MCS) from pORI28. Plasmid pTRK847 was designed as the platform for the development of the expression vectors containing the origin of replication and erythromycin resistance (Er^r) marker from pGK12, an MCS and two transcriptional terminators flanking the expression region. The terminators (Table 1) were inserted by ligating PCR products from primers TD_Term908F/R and TD_Term667F/R (Table 3) into the XbaI and BglII sites of the MCS respectively. Plasmids pTRK848, pTRK849 and pTRK850 contain the promoter and repressor from the *L. acidophilus* FOS (LBA0500-0507), lactose (LBA1465-1469) and trehalose (LBA1012-1014) operons. Also, pTRK882 contains the promoter for *pgm* (LBA0185) encoding phosphoglycerate mutase. These elements were obtained by PCR amplification and subsequently inserted into pTRK847 using restriction sites (see Table 2 for sources and Table 3 for primers).

To investigate expression from each promoter the *gusA3* gene (Callanan *et al*., 2007) encoding a modified β-glucuronidase (GusA3) was cloned into the expression region of each vector using the EcoRI and NotI sites in the MCS. In order to express the *L. acidophilus* oxalate gene operon (LBA0396-LBA0395) (Fig. 8A), primers OXF and OXR (Table 3) were used to amplify a 3.2 kb DNA fragment containing the *L. acidophilus* oxalate gene operon. This PCR product was cloned into pTRK882 using the EcoRI and NotI restriction sites. This plasmid appears in Fig. 8B.

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Table 3. PCR primers.

Primer	Sequence (5′ – 3′)*	Application
TD_pGK12Xba	ATGCTCTAGATTCAGGAATTGTCAGATAGG	Inverse PCR of pGK12, XbaI site
TD_pGK12Bgl	ATGCAGATCTACTAATGGGTGCTTTAGTTG	Inverse PCR of pGK12, BglII site
TD_Term908F	ATGCTCTAGATAATTCTATTGCTGAAACTG	Cloning of Term908
TD_Term908R	ATGCTCTAGAAGCTTATACACTGATAATAAC	Cloning of Term908
TD_Term667F	ATGCAGATCTTATTAAGATTACCGTTATCC	Cloning of Term667
TD_Term667R	ATGCAGATCTCTAATAAATGGCGTAATTG	Cloning of Term667
TD_PFOSE	ATGCGAATTCATTACTACAGCCAGTTAGTG	Amplification of P_{FOS}/*msmR* fragment
TD_PFOSN	ATGCCCATGGTTGATGAATAAGGTGAAGAAAG	Amplification of P_{FOS}/*msmR* fragment
TD_PLACE	ATGCGAATTCTTATGTTTGCTTGCATAC	Amplification of P_{lac}/*lacR* fragment
TD_PLACN	ATGCCCATGGATACTCTCGATACTTTATTAG	Amplification of P_{lac}/*lacR* fragment
TD_PTREE	ATGCGAATTCCACTTATTGGTGCAACAAC	Amplification of P_{tre}/*treR* fragment
TD_PTREN	ATGCCCATGGATCGCCATTTGAATCATAG	Amplification of P_{tre}/*treR* fragment
TD_PLA0185E	ATGCGAATTCAGCCTTCTTAGCTTCTTCAAC	Amplification of P_{pgm} fragment
TD_PLA0185N	ATGCCCATGGTGCGACAAGTAATAAACTAAAC	Amplification of P_{pgm} fragment
TD_OXF	ATGCGAATTCTCGTGGTTGATACATCACTC	Amplification of Lba 0395-Lba0396
TD_OXR	ATGCGCGGCCGCTTGGGTTCAGTCATTATGAC	Amplification of Lba 0395-Lba0396

*Restriction enzyme sites appear underlined.

GUS assays

β-Glucaronidase activity in CFEs was measured by the hydrolysis of 4-methyl-umbelliferyl-β-D-glucuronide (MUG) (Sigma). Cultures were grown to mid-log phase (A_{600} = 0.4–0.6) in MRS with 5 μg ml^{-1} Er, harvested by centrifugation at room temperature, washed with either MRS or SSM + 1% carbohydrate as indicated, resuspended in an equal volume of either MRS or SSM with 1% carbohydrate, and incubated at 37°C statically for 1–3 h. Cell-free extracts were prepared as previously described (Russell and Klaenhammer, 2001b). Protein concentrations were determined using the Sigma 96-well Assay (Sigma). For each assay, CFEs were warmed to 37°C, and 200 μl of CFE was added to 800 μl GUS buffer (100 mM sodium phosphate, 2.5 mM EDTA, 1.0 mM MUG, pH 6.0). After incubation at 37°C for 5 min, 1 ml of 0.2 M Na_2CO_3 was added. Activity is reported as picomoles of 4-methylumbelliferone liberated per minute per milligram of protein.

Oxalate degradation

Oxalate degradation was measured *in vitro* as described previously with the following modifications (Azcarate-Peril *et al*., 2006). *Lactobacillus* cultures were transferred three times in MRS with 0.01% (%w/v) ammonium oxalate (Fisher). Cultures were then inoculated at 1% into MRS with 0.01% ammonium oxalate and grown to OD_{600} = 0.5, centrifuged, and resuspended in MRS with 0.1% ammonium oxalate. Cultures were incubated statically at 37°C and samples were taken at 0, 24, 48, 72 and 96 h, centrifuged, neutralized to obtain pH values of 5–7 (according to the manufacturer's instructions) with 1 N sodium hydroxide, and stored at –20°C. The oxalate concentrations in the supernatants were determined using a diagnostic oxalate kit (Trinity Biotech, County Wicklow, Ireland).

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