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ORIGINAL ARTICLE

Dynamics of competitive population abundance of *Lactobacillus plantarum ivi* gene mutants in faecal samples after passage through the gastrointestinal tract of mice

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Abstract

Aim: This study aims to evaluate the impact of mutation of previously identified *in vivo*-induced (*ivi*) genes on the persistence and survival of *Lactobacillus plantarum* WCFS1 in the gastrointestinal (GI) tract of mice.
Methods and Results: Nine *Lact. plantarum ivi* gene replacement mutants were constructed, focussing on *ivi* genes that encode proteins with a predicted role in cell envelope functionality, stress response and regulation. The *in vitro* growth characteristics of the mutants appeared identical to those observed for the wild-type strain, which agrees with the recombination-based *in vivo* expression technology suggestion that these genes are not transcribed in the laboratory. Quantitative PCR experiments demonstrated differences in the relative population dynamics of the *Lact. plantarum ivi* mutants in faecal samples after passage through the GI tract of mice.
Conclusions: The *in situ* competition experiments revealed a 100- to 1000-fold reduction of the relative abundance of three of the *ivi* gene mutants, harbouring deletions of genes predicted to encode a copper transporter, an orphan IIC cellobiose PTS and a cell wall anchored extracellular protein.
Significance and Impact of the Study: These experiments clearly establish that the proteins encoded by these three genes play a key role in *Lact. plantarum* performance during passage of the GI tract.

Introduction

Three main strategies have been developed for the identification of genes that are either highly expressed, differentially expressed or specifically required *in vivo*, namely (recombination-based) *in vivo* expression technology [(R-)IVET], signature tagged mutagenesis and selective capture of transcribed sequences. The basic characteristics and relative (dis)advantages of these approaches have been addressed previously (Mahan *et al.* 2000; Angelichio and Camilli 2002) and will therefore not be discussed in detail here. Particularly, the different variants of the (R-)IVET approach have been applied to a large number of pathogenic bacteria, including *Escherichia coli*, *Salmonella enterica*, and *Klebsiella pneumoniae* (Mahan *et al.* 2000; Angelichio and Camilli 2002). The number of genes identified as *in vivo* induced (*ivi*) in these (R-)IVET screens is extremely variable and ranges from 1 to more than 100 (Mahan *et al.* 2000). Recently, the first IVET and R-IVET studies on the food-grade lactic acid bacteria *Lactobacillus reuteri* and *Lactobacillus plantarum* have been reported (Walter *et al.* 2003; Bron *et al.* 2004a). The *Lact. plantarum* strain used in the latter study had previously been shown to display particularly high activity and survival rates in the human intestine in pharmacokinetic experiments (Vesa *et al.* 2000). In addition, the complete genomic sequence of this strain is known, providing an advantageous knowledgebase that facilitates molecular analyses of the *in situ* behaviour of this bacterium in the gastrointestinal (GI) tract (Kleerebezem *et al.* 2003). Both *Lactobacillus* (R-)IVET studies explored the *in situ* gene expression of these lactobacilli in the mouse GI tract

when compared with laboratory conditions. Notably, the *Lact. reuteri* IVET study revealed 'only' three *ivi* genes in this species, while the R-IVET approach in *Lact. plantarum* revealed 72 *ivi* genes, illustrating the variation in the number of *ivi* genes identified in these types of studies. Intriguingly, a common *ivi* gene, encoding a conserved hypothetical protein, was identified in both lactobacilli (Walter *et al.* 2003; Bron *et al.* 2004a). Moreover, it was concluded that a significant parallel exists between *ivi* gene-functions revealed by R-IVET screening in *Lact. plantarum* and those identified by similar screening methods in pathogenic bacteria, suggesting that these functions are involved in intestinal persistence and survival rather than virulence or pathogenesis (Bron *et al.* 2004a).

Following the initial identification of *ivi* genes, quantitative reverse transcriptase PCR (qRT-PCR) approaches can be used to directly evaluate the *in situ* expression of (a subset of) these genes. In *Lact. plantarum* and *E. coli*, qRT-PCR experiments have revealed specific induction of gene expression in the duodenum and liver, respectively (Khan and Isaacson 2002; Bron *et al.* 2004b). Moreover, a more elaborate qRT-PCR analysis allowed refinement of the spatial and temporal expression patterns of 15 *ivi* genes of *Lact. plantarum* in the GI tract of mice (Marco *et al.* 2007). Another frequently applied follow-up strategy with *ivi* genes identified in pathogenic bacteria is the construction of gene deletion mutants followed by assessment of the relative virulence of the constructed mutants when compared with the wild-type strain (Mahan *et al.* 1993; Wang *et al.* 1996). In addition, several studies report the comparative abundance of the mutants in infected tissues relative to the wild-type strain, as determined by selective plate counts of both strains (Lowe *et al.* 1998; Gahan and Hill 2000; Gort and Miller 2000). These gene disruptions strategies have confirmed the importance of many *ivi* genes during the infection process of several pathogens, including *Staphylococcus aureus* (Lowe *et al.* 1998), *Pseudomonas aeruginosa* (Wang *et al.* 1996), serotype *Salmonella typhimurium* (Mahan *et al.* 2000), *Yersinia enterocolitica* (Gort and Miller 2000) and *Listeria monocytogenes* (Gahan and Hill 2000). In analogy, a recent study provided evidence that mutation of the methionine sulfoxide reductase identified by IVET in *Lact. reuteri* reduces the *in situ* performance of this bacterium in the mouse GI tract (Walter *et al.* 2005).

Here, we describe the construction of *Lact. plantarum* gene replacement mutants of nine of the 72 *ivi* genes that were identified using R-IVET. Growth of these mutants on MRS and chemically defined medium (CDM) was investigated, revealing that all mutants displayed virtually identical *in vitro* growth characteristics when compared with the wild-type strain. Equal amounts of cells of the individual chloramphenicol resistant mutants and a wild-type control strain (NZ7109) were administered to three mice and their relative population dynamics were investigated by quantitative PCR (qPCR). These experiments revealed that the relative abundance of several of the mutants was significantly reduced in faecal samples when compared with the initial mutant population ratios administered to the mice.

Material and methods

Bacterial strains, media and growth conditions

The parental strains and plasmids that were used in this study are listed in Table 1. The primers used for pNZ7101 constructions, control of chromosomal integration events and the primers and probes used during qPCR (see below) are listed in Table 2. The primers used during construction of the *Lact. plantarum cat*-replacement mutants and the constructed *Lact. plantarum* mutants are presented in Tables 3 and 4, respectively. *Escherichia coli* strain MC1061 (Casadaban and Cohen

Table 1 Strains and plasmids used in this study

Material	Relevant features	Reference
Strains		
Escherichia coli MC1061	Cloning host	Casadaban and Cohen (1980)
Lactobacillus plantarum WCFS1	Wild-type for which the genome sequence is available	Kleerebezem *et al.* (2003)
Lact. plantarum NZ7109	*Lact. plantarum* WCFS1 derivative containing chromosomal *ery* in a neutral locus	Bron *et al.* (2004a)
Plasmids		
pNZ84	Cm^R, pACYC184 derivative	van Alen-Boerrigter *et al.* (1991)
pGIZ850	Cm^R, contains P32-*cat* selectable as single copy in the chromosome of *Lact. plantarum*	Goffin and Hols, unpublished data
pUC18Ery	Amp^R Em^R	van Kranenburg *et al.* (1997)
pNZ7101	Cm^R Em^R, vector for construction of *Lact. plantarum* gene replacement mutants	This study

Amp^R, ampicillin resistant; Em^R, erythromycin resistant; Cm^R, chloramphenicol resistant.

Table 2 Primers used for pNZ7101 constructions, control of chromosomal integration events and primers and probe used during qPCR

Primer	Sequence
pNZ7101 construction	
pNZ84F	5′-CGGGATCCCAACAGTACTGCGATGAG-3′
pNZ84R	5′-GGGGTACCATCCAGTGATTTTTTTCTCC-3′
Integration controls	
PvuII-ori	5′-TAGTAAGCCAGTATACACTC-3′
SmaI- ori	5′-TTAAGAAGATACTGGCCGAA-3′
HTPcmF	5′-TAGTGACAAGGGTGATAAAC-3′
HTPcmR	5′-TCCTGACCACCATTATCAAG-3′
HTPeryF	5′-GATACCGTTTACGAAATTGG-3′
HTPeryR	5′-CCCATTTTGAAACAAAGTAC-3′
eryF	5′-AAGCAATGAAACACGCC-3′
PvuII- SCO	5′-TGATGGTGTTTTTGAGGTGC-3′
qPCR primers and probes	
uniF	5′-CCGAGCCTAAGGAACAGGC-3′
qPCR_0190	5′-AATTTGGGGGACCTACCGTAT-3′
qPCR_1019	5′-ACGACCATCTGCCTTAGGCT-3′
qPCR_1164	5′-CCTCCTCCTCAACCTGGTTT-3′
qPCR_1403	5′-TGGGTCACGATTGGCGCAAT-3′
qPCR_2940	5′-TAAGATAGTGATGGGAATCACC-3′
qPCR_3055	5′-ACTCGTGACTACGGACAATGA-3′
qPCR_3281	5′-TTTAAGCGGGATGACGTTGAC-3′
qPCR_3514	5′-TAATAATAACGTCCTATTGGCTG-3′
qPCR_3659	5′-ACGGTAACCGTTATTGGGAGT-3′
qPCR-MM	5′-FAM-CTAGAACAATTACGGCGCGATATGC-TAMRA-3′
TM-ery-F99	5′-TTCACCGAACACTAGGGTTGC-3′
TM-ery-R100	5′-CATTCCGCTGGCAGCTTAAG-3′
TM-ery-FAM101	5′-FAM-TGCACACTCAAGTCTCGATTCAGCA-TAMRA-3′

1980) was used as cloning host during construction of the pNZ7101-derived gene replacement vectors (see below). *Escherichia coli* was grown aerobically in TY medium (Sambrook *et al.* 1989). *Lactobacillus plantarum* WCFS1 (Kleerebezem *et al.* 2003) and the gene disruption mutants were grown at 37°C in MRS (Difco, Surrey, UK) or CDM (Poolman and Konings 1988) without aeration.

DNA techniques

Plasmid DNA was isolated from *E. coli* on a small scale using the alkaline lysis method (Birnboim and Doly 1979; Sambrook *et al.* 1989). Large-scale plasmid DNA isolations were performed using Jetstar columns according to the manufacturer's instructions (Genomed GmbH, Bad

Table 3 Primers used during construction of the *Lactobacillus plantarum cat*-replacement mutants

Target locus	Forward primer	Reverse primer
5′-lp_0190	5′-TACGAAAACTAAAGGC-3′	5′-ATGAACAACTACCGTTTAAC-3′
3′-lp_0190	5′-TCTTCAAATACGGTAGGTCC-3′	5′-TTGCCTTAACTAAGATCGCC-3
5′-lp_1019	5′-GGAGAAATTAGTCTCAGAAC-3′	5′-TGAAATATTTGGCCTGTTCC-3′
3′-lp_1019	5′-TTAAAACCAGCCTAAGGCAG-3′	5′-TGTAGTTGCATGTCAGGTTG-3′
5′-lp_1164	5′-TCATAGCACAACTGGATAGG-3′	5′-GACCCGTTCAAAGGTCAATA-3′
3′-lp_1164	5′-TTGGCAAACCAGGTTGAGGA-3′	5′-CGTTTCAGCCAGTAAGTACT-3′
5′-lp_1403	5′-CTTTGACCATCGTTTTAGCA-3′	5′-TGGCTCTATGAAAACATCTC-3′
3′-lp_1403	5′-TAAGCTAATTGCGCCAATCG-3′	5′-GATTTTGCGACTTGGCAGAC-3′
5′-lp_2940	5′-GCTAAACTAAAAAAACGGTCG-3′	5′-GGTTTTAGCAAGCAACAACG-3′
3′-lp_2940	5′-TAACATGGTGATTCCCATCA-3′	5′-TATCAGGATGCTGAGTATCA-3′
5′-lp_3055	5′-TCCCAAGCGACGTAAATCTA-3′	5′-GTAGTCACGAGTTCGGTATTG-3′
3′-lp_3055	5′-ATCATATTAGCCAGTAGTGG-3′	5′-GAGTGCGGTTGATGATTTAC-3′
5′-lp_3281	5′-CGATTAAAGTACCAGACGAC-3′	5′-GTGACGTCTCAAATAGCGAA-3′
3′-lp_3281	5′-GTACAAGTCAACGTCATCCC-3′	5′-CGCGACTTTGCCAATAAGTT-3′
5′-lp_3514	5′-ACGACAATACCCAAACTGCC-3′	5′-ATCATGCACATTCAGCGGAT-3′
3′-lp_3514	5′-TTCAGCCAATAGGACGTTAT-3′	5′-AAGGAACAGTTAGAATCACG-3′
5′-lp_3659	5′-TTGGTAGTCTTCTTTTGGCC-3′	5′-ACTGGTGAAATGACACCTTA-3′
3′-lp_3660	5′-GTTGATACTCCCAATAACGG-3′	5′-GAATCCCTTTTTTGGTGAGC-3′

Table 4 Overview of the constructed *Lactobacillus plantarum* mutant strains, the functions of the replaced open reading frames (ORFs) and their maximal growth rate on MRS and CDM media

Strain	Replaced ORF(s) (genes)	Removed function(s)	μ_{max} (h^{-1}) on MRS	μ_{max} (h^{-1}) on CDM
Lact. plantarum WCFS1			0·80 ± 0·02	0·19 ± 0·02
Lact. plantarum Δlp_0190	lp_0190	conserved hypothetical	0·83 ± 0·17	0·17 ± 0·01
Lact. plantarum Δlp_1019	lp_1019 (*clpC*)	ATP-dependent Clp protease	0·76 ± 0·11	0·18 ± 0·01
Lact. plantarum Δlp_1164	lp_1164 (*pts14C*)	cellobiose PTS, EIIC	0·87 ± 0·03	0·21 ± 0·01
Lact. plantarum Δlp_1403	lp_1403	cell surface protein	0·87 ± 0·14	0·22 ± 0·02
Lact. plantarum Δlp_2940	lp_2940	cell surface protein precursor	0·91 ± 0·04	0·23 ± 0·01
Lact. plantarum Δlp_3055*	lp_3055 (*copA*)	copper transporting ATPase	0·84 ± 0·08	0·22 ± 0·01
Lact. plantarum Δlp_3281	lp_3281	transport protein	0·90 ± 0·16	0·19 ± 0·02
Lact. plantarum Δlp_3514	lp_3514 (*bglG4*)	transcription antitermination	0·85 ± 0·05	0·21 ± 0·01
Lact. plantarum Δlp_3659	lp_3659 (*rbsD*) and lp_3660 (*rbsK3*)	ribose transport protein and ribokinase	0·81 ± 0·04	0·21 ± 0·02

*In this strain, the chloramphenicol gene is transcribed in the opposite orientation relative to the original gene.

Oberhausen, Germany). DNA isolation and transformation in *Lact. plantarum* were performed as described previously (Josson *et al.* 1989; Ferain *et al.* 1994). Standard procedures were applied for DNA manipulations in *E. coli* (Sambrook *et al.* 1989). Restriction endonucleases, *Taq* and *Pfx* polymerase, and high-concentration T4 DNA ligase were used following the recommendations of the manufacturer (Promega, Leiden, the Netherlands and Boehringer, Mannheim, Germany). Primers were purchased from Pharmacia Biotech (Roosendaal, the Netherlands).

Plasmid constructions and gene replacements in *Lactobacillus plantarum*

A universal and efficient method was employed for the construction of gene replacement mutants in *Lact. plantarum*. For this purpose, the vector pNZ7101 was constructed that allows single-step selection of *Lact. plantarum* gene replacement mutants. The pACYC184-derived origin of replication (Rose 1988) was PCR amplified as a 2·0-kb fragment using *Pfx* polymerase, with the primers pNZ84F and pNZ84R (Table 2), and pNZ84 (van Alen-Boerrigter *et al.* 1991) as a template. The obtained amplicon was ligated with the 1·5 kb *Nae*I fragment of pGIZ850 (Philippe Goffin and Pascal Hols, UCL laboratory collection, Louvain, Belgium, unpublished data) that contains the *cat*-194 gene (Horinouchi and Weisblum 1982) under control of the lactococcal P32 promoter (van der Vossen *et al.* 1987). The resulting plasmid was digested with *Sma*I and *Sph*I, and ligated to a 1·1 kb *Sma*I-*Sph*I fragment of pUC18Ery (van Kranenburg *et al.* 1997), which contains the erythromycin-resistance encoding *ery* gene. The resulting vector was designated pNZ7101 and is a suitable mutagenesis vector that harbours two selectable markers (*ery* and *cat*) that can both be selected at single-copy expression level in the *Lact. plantarum* chromosome (Fig. 1).

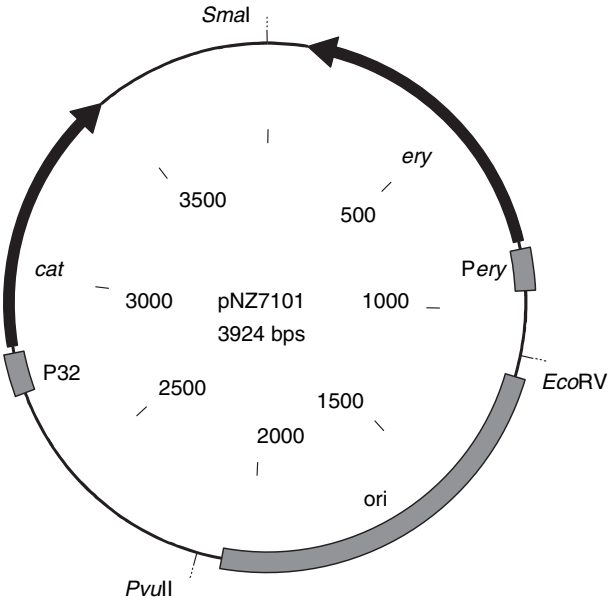

Figure 1 Schematic representation of the mutagenesis vector pNZ7101 that allows single-step selection of *cat*-gene replacement mutants in *Lactobacillus plantarum*.

pNZ7101 was utilized for the construction of nine *cat*-replacement mutants in *Lact. plantarum* WCFS1. As a first step, the 18 5′- and 3′-flanking regions of the nine target genes were amplified using *Pfx* polymerase, locus-specific primers (Table 3) and chromosomal DNA of *Lact. plantarum* WCFS1 as template. The resulting amplicons were glass matrix purified (Amersham Biosciences, Roosendaal, the Netherlands) and the 5′-flanking regions were cloned in *Pvu*II digested pNZ7101. The ligation mixtures were digested with *Pvu*II prior to transformation to

E. coli MC1061 (Casadaban and Cohen 1980) to reduce the number of transformants harbouring backligated vector DNA. After 48 h, full-grown colonies were used directly as templates in a PCR reaction using primer PvuII-ori and the corresponding mutant-specific 5′R primer (Tables 2 and 3, respectively). This PCR reaction only generates a product when an insert is cloned in the appropriate orientation. Using this approach, nine clones were selected that harbour a PNZ7101 derivative containing the gene-specific 5′-flanking region. Plasmid DNA derived from these clones was digested with *Sma*I and ligated to the PCR product of the corresponding 3′-flanking region. The procedure used was essentially the same as that described for the 5′-flanking regions, including *Sma*I digestion of the ligation mixtures prior to transformation, and orientation-specific PCR-based clone selection using primers SmaI-ori and the locus-specific 3′R primer (Tables 2 and 3, respectively). This approach resulted in the construction of eight plasmids that harbour the chloramphenicol resistance gene flanked by the 5′- and 3′-flanking regions of eight *ivi* target genes. For the target gene lp_3055 cloning of the 3′-flanking region was unsuccessful. Therefore, the 5′- and 3′-flanking regions of this gene were cloned in pNZ7101 in the opposite orientation (5′- and 3′-flanking regions cloned in *Sma*I and *Pvu*II, respectively). Using this approach, the *cat*-gene replacement vector for lp_3055 could be obtained. Notably, all nine plasmids constructed were subjected to extensive restriction and PCR analyses, confirming their anticipated organization (data not shown).

A double cross-over chloramphenicol replacement strategy was used for the stable *cat*-gene replacement of the nine selected *ivi* genes. The mutagenesis plasmids were transformed to *Lact. plantarum* WCFS1 and integrants were selected on MRS plates containing 5 μg chloramphenicol ml^{-1}. After 48 h, full-grown colonies were replica-plated to MRS plates containing 5 μg chloramphenicol ml^{-1}, with or without 30 μg ml^{-1} erythromycin. Colonies displaying an erythromycin-sensitive phenotype represent candidate *cat*-gene replacements that result from a simultaneous double cross-over event in both the 5′- and the 3′-flanking regions of the *ivi* gene. The anticipated *cat*-replacement genotype of these candidate mutants was verified by five PCR reactions using chromosomal DNA of the mutants as template. These reactions include a multiplex PCR using the primer pairs HTPcmF plus HTPcmR and HTPeryF plus HTPeryR to confirm the presence of the *cat* gene and the absence of the *ery* gene in the chromosomes of the mutants, respectively (Table 2). Moreover, the absence of plasmid-derived DNA sequences was investigated using the primer combinations SmaI-ori and eryF, and PvuII-ori and PvuII-SCO (Table 2). Finally, the conformation of the *cat*-integration loci was checked using the primer pairs PvuII-ori combined with the corresponding mutant-specific 5′R primer, and SmaI-ori combined with the corresponding 3′R primer (Tables 2 and 3). Using these PCR reactions, the anticipated genotype of the nine *cat*-replacement mutant strains could be confirmed (Table 4).

Maximal growth rate determination

The maximal growth rates of *Lact. plantarum* WCFS1 and its *cat*-replacement mutant derivatives on MRS and CDM (Poolman and Konings 1988) were determined. Full-grown cultures were diluted 100-fold in fresh media in a 96-well plate and growth was monitored by automatic OD_{600} measurements using a SPECTRAmax® PLUS^{384} microplate spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA, USA). All experiments were performed in triplicate and the obtained data were used to calculate the maximal logarithmic growth rate of all strains in MRS and CDM.

Animal experiments

A method was developed to monitor the relative population dynamics of the group of *Lact. plantarum cat*-replacement mutants in faecal samples after passage through the GI tract, in competition with each other and the wild-type control strain NZ7109. Notably, NZ7109 harbours an erythromycin resistance marker at a neutral locus in its chromosome (Bron *et al.* 2004a), allowing its incorporation in these experiments as a wild-type control strain. Equal amounts of overnight cultures of all bacterial strains were mixed, pelleted by centrifugation and resuspended at approx. 10^{10} CFU (total population) per millilitre in MRS. Three female Balb/c mice (Iffa Credo, St Germain sur l'Arbresle, France), which had free access to tap water and standard mice chow during the course of the experiments, received a 100-μl oral dose of the bacterial suspension by intragastric administration at two consecutive days (day -1 and 0). Individual faecal samples were collected daily during 6 days following the last bacterial administration (day 1–6) and the samples were stored at −80°C until they were further processed.

DNA isolation from faecal samples

DNA was isolated from the mixed input populations administered to the mice, and from faecal samples essentially as described before (Zoetendal *et al.* 2001). Approx. 10^8 CFU from the mixed input population or 0·1 g of faecal sample were resuspended in 0·4 ml TE buffer (10 mmol l^{-1} Tris-HCl, 1 mmol l^{-1} EDTA, pH 8·0) containing 10 μg RNase ml^{-1}. After thorough homogeniza-

tion, 50 μl 10% SDS and 10 μl 20 mg proteinase K ml^{-1} solution were added, and the resulting suspensions were incubated for 1 h at 65°C. Subsequently, 0·5 g of zirconium glass beads, 0·5 ml phenol and 200 μl chloroform were added and the cells were disrupted using two treatments of 40 s in a Fastprep™ (Qbiogene Inc, Cedex, France) interspaced by 1 min on ice. After centrifugation, the aqueous phase was used for DNA isolation using several phenol–chloroform extractions, followed by precipitation of the DNA using 1 volume of isopropanol. Subsequently, the pellets were washed with 70% (v/v) ethanol, vacuum-dried and dissolved in 200 μl TE buffer by overnight incubation at 4°C.

Quantitative PCR analysis

To compare the relative *in vivo* abundance of all constructed *Lact. plantarum cat*-replacement mutants in the faecal samples, a mutant-specific qPCR was performed using DNA derived from mixed input populations and faecal samples at different time points as template. Each 50-μl amplification reaction contained 5 μl of the isolated total DNA, 1× Taqman Universal PCR master mix (Applied Biosystems, Nieuwekerk a/d IJssel, the Netherlands), 200 nmol l^{-1} of uniF primer, mutant-specific qPCR primer and the Taqman qPCR-MM probe (Table 2). Reactions were performed using an ABI Prism 7700 PCR machine (Applied Biosystems). Reactions were initiated at 95°C for 10 min, followed by 40 amplification cycles of 95°C for 15 s and 55°C for 60 s. Cycle threshold (C_t) values were obtained upon manual setting of the baseline at a threshold value at which fluorescence was appreciably above background fluorescence for each reaction and within the exponential phase of amplification for all reactions. To assess the abundance of the *ery*-tagged control strain NZ7109, essentially the same qPCR conditions were used. However, each reaction contained 200 nmol l^{-1} of primers TM-ery-F99, TM-ery-R100 and the Taqman probe TM-ery-FAM101 (Table 2). All reactions were performed in triplicate and the reciprocal of the 2-power of the C_t values was used as a measure for the absolute amount of mutant-specific templates. The mutant-specific population measurements were normalized using the abundance number obtained for the NZ7109 control strain.

Results

pNZ7101 and its employment for the construction of nine *Lactobacillus plantarum ivi* gene replacement mutants

Previously, we have described the identification of 72 *Lact. plantarum* genes that are induced during GI tract passage in mice when compared with laboratory conditions (Bron *et al.* 2004a). Here, nine of these *ivi* genes were selected for gene disruption and further analyses. The majority of the selected genes encodes proteins with a predicted role in cell envelope functionality (lp_1164, lp_1403, lp_2940, lp_3281 and lp_3659), stress response (lp_1019 and lp_3055) or regulation (lp_3514). Moreover, one gene was selected that encodes a protein for which no function could be predicted and encodes a conserved hypothetical protein (lp_0190). To allow gene disruption and subsequent further establishment of a role for these genes in the observed GI persistence of *Lact. plantarum*, the gene replacement vector pNZ7101 was constructed (Fig. 1). This vector has a relatively low copy number in *E. coli*, does not replicate in Gram-positive bacteria, and harbours two antibiotic selection markers (*ery* and *cat*), which are flanked by blunt-end restriction sites. Amplicons harbouring the flanking regions of the target *Lact. plantarum* genes were generated using a proof-reading polymerase, and were cloned directly in the blunt-end restriction sites of pNZ7101 (see 'Material and methods' and Fig. 2). Subsequently, the constructed pNZ7101 derivatives were transformed to *Lact. plantarum* and the *ivi* gene replacement mutants, resulting from a double cross-over event, were identified on the basis of their antibiotic resistance phenotype (Cm^R and Em^S) and the corresponding anticipated chromosomal organization of the mutation loci was confirmed by PCR (see 'Materials and methods').

In vitro growth characteristics of the *Lactobacillus plantarum ivi* gene replacement mutants

Initially, the growth characteristics of the nine *Lact. plantarum cat*-replacement mutants were investigated *in vitro* on MRS and CDM medium (Table 4). These experiments revealed that the maximal growth rate of all mutants was not significantly different when compared with that observed for the wild-type strain on both types of media. Moreover, the final OD_{600} reached by all strains was virtually identical (data not shown). The fact that the genes mutated in these *Lact. plantarum* WCFS1 derivatives were selected by R-IVET screening implies that these genes are not expressed under laboratory conditions (Bron *et al.* 2004a). Therefore, the finding that mutation of these genes did not result in dramatic effects on growth under laboratory conditions was not unexpected and is in agreement with the R-IVET results.

Relative population dynamics in faecal samples after gastrointestinal tract passage

To assess their relative abundance in faecal samples after competitive passage through the GI tract of mice, the

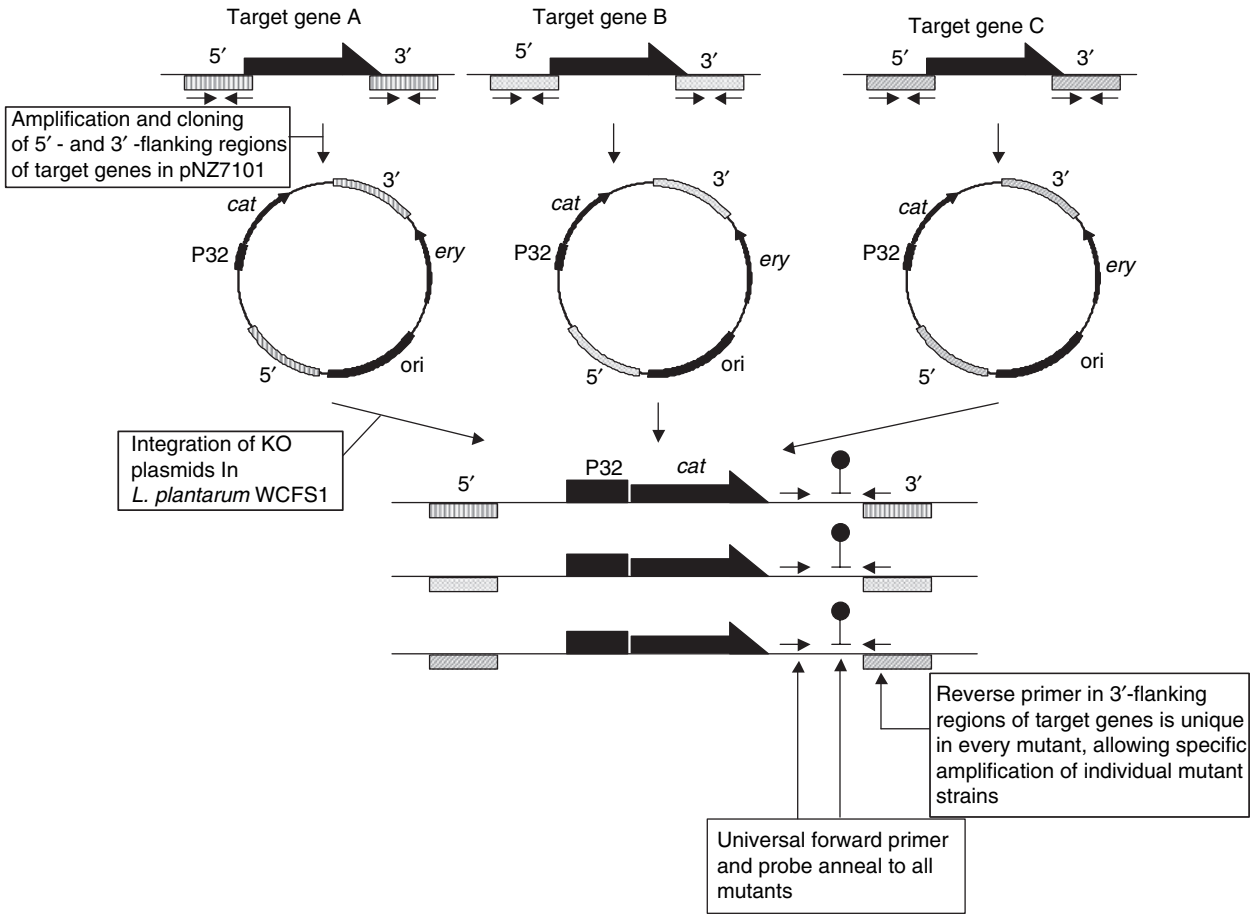

Figure 2 Schematic overview of the strategy that was employed to construct multiple *cat*-replacement mutants in *Lactobacillus plantarum*, and the design of a qPCR approach that allows quantitative amplification of individual mutant strains.

nine *ivi* mutants and the NZ7109 *ery*-tagged control strain (Bron *et al.* 2004a) were intragastrically administered in equal amounts to three mice on two consecutive days (day -1 and 0), and faecal samples were collected during the following 6 days (day 1–6). A qPCR strategy was developed using total DNA isolated from the faecal samples as template, allowing assessment of the abundance of the control strain NZ7109 by using primers and a probe specific for the *ery* gene. The abundance numbers obtained for the NZ7109 control strain were used to normalize the abundance numbers generated for the mutants (see below).

The qPCR procedure employed for the mutants generates a specific amplicon for every mutant by using one mutant-specific primer in combination with a universal primer and probe (Fig. 2). *In vitro* control experiments demonstrated that a primer combination designed to specifically amplify a single *ivi* mutant did not generate a PCR product when chromosomal DNA of any of the other mutants was used as template. This lack of cross-amplification establishes the high specificity of this mutant-specific qPCR approach (data not shown). Moreover, no detectable signal was obtained when DNA isolated from faecal samples derived from a mouse that had not been fed with *Lact. plantarum* WCFS1 was used as template in any of the qPCR reactions described above. These experiments demonstrate the absence of any background template in the control faecal samples and eliminate the possibilities for qPCR-signal interference because of PCR artefacts (data not shown). Furthermore, a dilution range of the nine mutant-specific pNZ7101 derivatives was used as template in the mutant-specific reactions. The obtained C_t values were used to calculate the PCR efficiency for all nine mutant-specific reactions, revealing PCR efficiencies ranging from 1·90 to 2·05. The fact that all PCR reactions have a relatively similar efficiency allows direct calculation of the relative abundance of all individual mutants from the obtained C_t values. In addition, extrapolation of the data obtained from the template dilution ranges allowed the estimation of

template detection limits, which appeared to be similar for all mutant loci and was concluded to be below 20 template copies for all loci (data not shown).

The qPCR data obtained clearly demonstrated that the input population size of individual mutants present in the mixtures administered to the mice on day -1 and 0 are not significantly different (Fig. 3). Furthermore, the normalized, relative abundance of some of the mutants in the faecal samples obtained on day 1–6 from all three mice appeared to be consistently and significantly lower when compared with other mutant strains. This finding is clearly exemplified by the finding that the relative abundance of the mutant strains Δlp_1164, Δlp_2940 and Δlp_3055 (Fig. 3c) appeared to be 100- to 1000-fold lower when compared with the mutant strains Δlp_1403, Δlp_3281 and Δlp_3659 (Fig. 3a). The latter mutants are representatives of the group of strains that displayed the highest relative abundance, which is comparable to the abundance of the wild-type. Although the abundance reduction-factor for *Lact. plantarum* Δlp_1164, Δlp_2940 and Δlp_3055 appeared to be slightly variable between mice, comparative analysis in individual mice consistently revealed that these *ivi* mutants displayed the strongest abundance reduction in all mice. Finally, three mutant strains, Δlp_0190, Δlp_1019 and Δlp_3514, appeared to display intermediary abundance in the faecal samples analysed. A more significant assessment of the relative abundance of this group of *ivi* mutants probably requires expansion of the number of measurements and mice. Nevertheless, these experiments demonstrate the suitability of locus-specific qPCR to assess and compare the abundance of multiple mutants in a single sample, and clearly establish the reduced abundance of the Δlp_1164, Δlp_2940 and Δlp_3055 *Lact. plantarum* mutants in the faecal samples. Moreover, the reduced abundance of these three mutants supports a critical role for the disrupted genes in GI tract survival and/or persistence of *Lact. plantarum*.

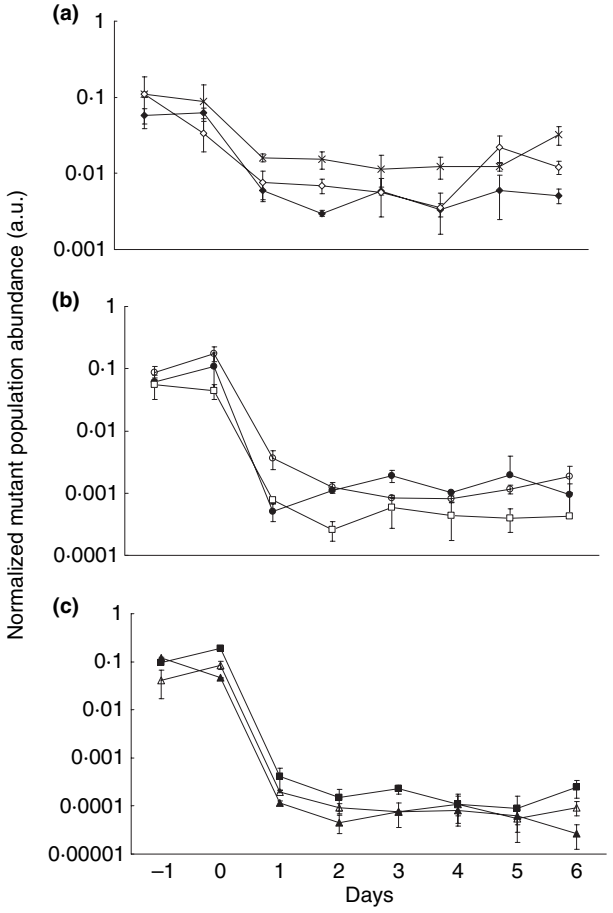

Figure 3 Determination of the relative abundance of individual mutants relative to the wild-type strain NZ7109. The abundance of individual mutants is represented as the reciprocal 2-power of the obtained C_t values, normalized by the abundance score obtained for the wild-type control strain NZ7109. Days 1 and 0 represent evaluation of relative population sizes of individual mutants within the mixture administered to the mice, whereas days 1–6 represent those encountered within faecal samples collected on the 6 days following the last administration. The mutants analysed were divided in three groups according to their relative abundance. (a) The relative abundance of the mutants displaying the highest abundance; Δlp_1403 (closed diamonds), Δlp_3659 (open diamonds) and Δlp_3281 (crosses). (b) The intermediary abundance group of mutants; Δlp_1019 (closed circles), Δlp_0190 (open circles) and Δlp_3514 (open squares). (c) The group of mutants with the lowest relative abundance; Δlp_2940 (closed triangles), Δlp_1164 (open triangles) and Δlp_3055 (closed squares). The error bars represent the standard deviation in independent, triplicate qPCR determinations.

Discussion

This paper describes the construction of pNZ7101; a low copy number *E. coli* cloning vector for the universal and efficient construction of mutagenesis vectors that allow single-step selection of double-cross over gene replacement mutants in *Lact. plantarum*. Subsequently, pNZ7101 was employed to construct nine isogenic *Lact. plantarum* WCFS1 gene replacement mutants. These mutations were introduced in genes that were previously demonstrated to be *in vivo* induced (*ivi*) during passage of the mouse GI tract (Bron *et al.* 2004a). The growth characteristics of all mutants on two laboratory media (MRS and CDM) were virtually identical to those of the wild-type strain. Furthermore, a qPCR approach was developed, focusing on the quantitative and specific detection of individual *ivi* mutants that are present in a mixture of all mutants and a wild-type strain. This method was employed to assess the normalized, relative population dynamics of individual *Lact. plantarum* *ivi* mutants in faecal samples after competitive passage through the GI tract of mice. This approach revealed that

in particular the normalized abundances of *Lact. plantarum* Δlp_1164, *Lact. plantarum* Δlp_2940 and *Lact. plantarum* Δlp_3055 were significantly and consistently decreased in three mice. In contrast, the normalized, relative abundance of three other mutants appeared to be similar to that of the wild-type (Δlp_1403, Δlp_3281 and Δlp_3659), while the last three mutants displayed an intermediary level of reduction of their relative population abundance after mouse passage (Δlp_0190, Δlp_1019 and Δlp_3514). Hence, these data clearly demonstrate that lp_1164, lp_2940 and lp_3055 play a key role in the functionality of *Lact. plantarum* during passage of the GI-tract.

The IIC transport component of the cellobiose PTS system predicted to be encoded by lp_1164 is not located in a typical PTS-operon structure. Notably, the best homologues of lp_1164 in the Genbank and ERGO databases (http://www.ncbi.nlm.nih.gov/ and http://ergo.integratedgenomics.com/ERGO/, respectively) include several other so-called orphan PTS-IIC transport components in other bacteria. Moreover, in *Streptococcus thermophilus*, *Pediococcus pentosaceus*, *L. johnsonii* and *L. casei*, the genomic organization of these homologues appears to be highly similar to the lp_1164 locus in *Lact. plantarum*, as the downstream gene encodes an acetyltransferase/hydrolase with a typical $\alpha\beta$-hydrolase fold in all these organisms. Importantly, in *L. monocytogenes* orphan cellobiose-PTS-IIC components have an established role in host-specific signalling, leading to modulation of virulence gene expression (Kreft and Vazquez-Boland 2001). Notably, many of these PTS systems are absent in *Listeria innocua* (Glaser *et al.* 2001). These findings, together with the experiments presented here suggest that host-factor-mediated gene regulation in bacteria could involve analogous bacterial signalling factors (orphan cellobiose PTS-IIC components) in different bacteria, including *Lact. plantarum*.

The lp_2940 gene is predicted to encode an extracellular protein that contains an LPQTNE motif involved in sortase-dependent anchoring of this protein to the bacterial cell wall (Mazmanian *et al.* 1999; Kleerebezem *et al.* 2003; Boekhorst *et al.* 2005). The bacterial surface is the primary site of interaction with the host and the protein encoded by lp_2940 might represent a factor that mediates interaction with a (host-derived) GI-tract-specific factor, or with host epithelial cells. Notably, the protein encoded by lp_2940 has no homologues in the Genbank and ERGO databases (data not shown). Moreover, no recognizable sequence motifs or domains could be identified in this protein. Therefore, it remains to be established what the exact role of this protein in *Lact. plantarum* is.

lp_3055 is predicted to encode a copper-transporting ATPase and could be involved in copper acquisition. Alternatively, this transporter could act as an exporter; thereby preventing accumulation of copper in the cytoplasm. Notably, heavy metal transport is a common theme that arose from several IVET screens in pathogens (Mahan *et al.* 2000), and copper homeostasis has been recognized in an IVET screen as important during the infection process of *Salm. typhimurium* (Heithoff *et al.* 1997). Furthermore, among the best homologues of *Lact. Plantarum*, CopA is the CtpA protein of *L. monocytogenes* (56% identity), which has an established role in copper transport. Gene expression studies in *L. monocytogenes* suggest that CtpA functions as both detoxification and acquisition mechanism, as the expression of *ctpA* is induced by copper limitation and high copper concentrations (Francis and Thomas 1997a). Growth of *L. monocytogenes ctpA* mutants was restricted by copper-chelating agents (Francis and Thomas 1997a). Moreover, recovery of *ctpA* mutants from tissue of infected mice was dramatically reduced when compared with the wild-type strain, and a significant impairment in terms of *in vivo* persistence in mixed-infection competition experiments was observed (Francis and Thomas 1997b). Our results suggest a similar role in *in vivo* persistence for CopA in *Lact. plantarum*, which is most probably related to the same *in situ* function of CopA and CtpA in the two species. Whether the *in situ* importance of these proteins relates to copper detoxification or copper acquisition remains to be established.

Several papers describe the construction of *ivi* gene deletion mutants in pathogenic bacteria, followed by assessment of the abundance of individual mutants when compared with the wild-type strain. Notably, these experiments involve selective plate counts of mutant and wild-type strains and are usually performed with bacterial cells that are in the post-invasion stage, e.g. isolated from infected tissues or organs (Lowe *et al.* 1998; Gahan and Hill 2000; Gort and Miller 2000). These plate counts generally do not allow the differential enumeration of more than one mutant in a single experiment and, therefore, can only be performed in binary experiments comparing an individual mutant with the wild-type strain. As a consequence, relatively large numbers of laboratory animals are required to compare multiple mutants with each other and/or a wild-type strain. In contrast, the qPCR approach described here is a culture-independent approach and allows the assessment of groups of mutants in complex competition experiments, generating a large amount of relative abundance data for individual mutants using only limited numbers of laboratory animals. To our knowledge, this is the first paper evaluating the *in situ* abundance of multiple isogenic mutants of a food-grade bacterium in a competitive system using a differential qPCR approach. Following the competitive population

dynamics experiments presented here, selected mutant strains can be analysed in binary experiments with the wild-type *Lact. plantarum* to generate a more detailed view of these mutants. Such experiments could include qPCR using total DNA derived from different locations in the GI tract, e.g. stomach, small intestine or colon, combined with selective enumeration of mutant and wild-type viable cells. Eventually, such experiments might reveal the molecular nature of the environmental conditions responsible for the relatively low abundance of specific mutants and the induction of the corresponding gene in the wild-type strain, which would provide the knowledge base that is required to understand the GI tract behaviour of *Lact. plantarum* and related species at the molecular level.

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