RESEARCH LETTER

An efficient and reproducible method for transformation of genetically recalcitrant bifidobacteria

Fausta Serafini[1], Francesca Turroni[2], Simone Guglielmetti[3], Laura Gioiosa[4], Elena Foroni[1], Valentina Sanghez[4], Alessandro Bartolomucci[5], Mary O'Connell Motherway[2], Paola Palanza[4], Douwe van Sinderen[2] & Marco Ventura[1]

[1]Laboratory of Probiogenomics, Department of Genetics, Biology of Microorganisms, Anthropology and Evolution, University of Parma, Parma, Italy; [2]Alimentary Pharmabiotic Centre and Department of Microbiology, Bioscience Institute, National University of Ireland, Cork, Ireland; [3]Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche, Università degli Studi di Milano, Milan, Italy; [4]Department of Evolutionary and Functional Biology, University of Parma, Parma, Italy; and [5]Department of Integrative Biology and Physiology, University of Minnesota, Minneapolis, MN, USA

Correspondence: Marco Ventura, Laboratory of Probiogenomics, Department of Genetics, Biology of Microorganisms, Anthropology and Evolution, University of Parma, Parco Area delle Scienze 11a, 43100 Parma, Italy. Tel.: +39 521 905666; fax: +39 521 906014; e-mail: marco.ventura@unipr.it

Received 5 April 2012; revised 18 May 2012; accepted 23 May 2012.
Final version published online 19 June 2012.

DOI: 10.1111/j.1574-6968.2012.02605.x

Editor: Ezio Ricca

Keywords
bifidobacteria; genomics; transformation; genetic manipulation.

Abstract

This study describes an efficient transformation system for the introduction of plasmid DNA into *Bifidobacterium bifidum* PRL2010 and *Bifidobacterium asteroides* PRL2011, for which to the best of our knowledge no transformation data have been reported previously. The method is based on electroporation of bifidobacterial cells, which were made competent by an optimized methodology based on varying media and growth conditions. Furthermore, the transformation protocol was applied in order to design a PRL2010-derivative, which carries antibiotic resistance against chloramphenicol and which was used to monitor PRL2010 colonization in a murine model.

Introduction

Bifidobacteria are Gram-positive G+C%-rich, anaerobic/microaerophilic, fermentative bacteria, which are often Y- or V-shaped (Ventura *et al.*, 2007). *Bifidobacterium* represents one of the most numerically abundant bacterial genera of the human gut microbiota in infants and is presumed to play a fundamental role in host health, which drives their wide-spread use as probiotic bacteria in many functional foods. This commercial exploitation of probiotic bifidobacterial strains has fuelled scientific interest in these bacteria to identify the genomic traits that are responsible for the claimed beneficial activities.

To exploit the full potential of these microorganisms for applications as probiotic ingredients, further knowledge is required on their molecular biology and genetics. However, molecular studies of *Bifidobacterium* are severely hampered by the absence of effective genetic tools, including efficient transformation protocols. So far, several *Bifidobacterium* strains, including members of *Bifidobacterium bifidum* and *Bifidobacterium asteroides*, have been shown to be non-transformable or very poorly transformable (Argnani *et al.*, 1996). Many factors may contribute to bifidobacterial recalcitrance for acquiring exogenous DNA, such as the presence of a thick (multilayered) and complex cell wall (Fischer *et al.*, 1987), intracellular restriction/modification barriers (Hartke *et al.*, 1996; Schell *et al.*, 2002; O'Connell Motherway *et al.*, 2009), and sensitivity to environmental stresses, in particular oxygen, to which these strictly anaerobic bacteria are exposed to during the preparation of competent cells and transformation procedure.

With the advent of the genomics era, many bifidobacterial genomes have been fully decoded (for reviews, see Turroni *et al.*, 2011; Ventura *et al.*, 2009), which has thus

provided a huge amount of genetic data that can be exploited to study genome functionality. Such studies are needed to understand the molecular mechanisms sustaining the interaction of bifidobacteria with its host as well as with other members of the gut microbiota (Hartke *et al.*, 1996; Schell *et al.*, 2002; Sela *et al.*, 2008; Ventura *et al.*, 2009; Turroni *et al.*, 2011).

However, to perform such functional genomic investigations, it will be necessary to develop transformation protocols as well as to implement gene knock-out methodologies effective for bifidobacteria. In this report, we describe the development of a protocol for efficient and reproducible genetic transformation of *B. bifidum* PRL2010 by electroporation using the shuttle vector pNZ8048 (de Ruyter *et al.*, 1996). The protocol of transformation is based on the preparation of electro-competent cells and subsequent electroporation and on the optimization of several parameters such as growth conditions, washing solutions, and electroporation voltage.

Materials and methods

Bacterial strains and plasmids

The *Bifidobacterium* strains used are described in Table 1. Plasmid pNZ8048 is a broad-host shuttle vector, which possesses the nisin-inducible *nisA* promoter and a chloramphenicol resistance gene as the selection marker (de Ruyter *et al.*, 1996).

Media and growth conditions

Escherichia coli strain DH10B, used as host strain for propagating the shuttle vector, was cultivated in LB medium (Savino *et al.*, 2011) supplemented with chloramphenicol (Sigma) at a final concentration of 10 μg mL^{-1}. The susceptibility to chloramphenicol of the bifidobacterial strains PRL2010 and PRL2011 was tested by means of a Minimal Inhibitor Concentration (MIC) assay, according to a previously described procedure (Serafini *et al.*, 2011).

Bifidobacteria were cultivated in de Man–Rogosa–Sharpe (MRS) medium supplemented with 0.05% cysteine-HCl (cMRS) in an anaerobic chamber (Concept 400, Ruskin; 2.99% H_2, 17.01% CO_2 and 80% N_2) at 37 °C for 24–72 h. In case of cultivation of bifidobacterial transformants, chloramphenicol was added to the growth medium cMRS agar at a final concentration of 3 μg mL^{-1}.

Table 1. Bacterial strains and plasmids used in this study

Bacterial strains		Reference
B. bifidum PRL2010		Turroni *et al.* (2010)
B. asteroides PRL2011		Unpublished data
Plasmids		
Code	Size (bp)	
pNZ8048	3349 bp	de Ruyter *et al.* (1996)

DNA isolation procedures

Plasmid DNA was isolated from *E. coli* as well as from bifidobacterial transformants using a Qiagen Plasmid Mini Kit. For Bifidobacteria, an additional incubation step in 20 mg mL^{-1} lysozyme at 37 °C for 40 min was performed before beginning the Qiagen kit protocol (Guglielmetti *et al.*, 2008).

Preparation of bacteria for electroporation

An overnight culture of *Bifidobacterium* (10%) was used to inoculate fresh MRS broth supplemented with 0.05% (final concentration) cysteine-HCl and 16% (v/w) fructo-oligosaccharides (FOS) (Actilight®; Beneo-Orafti), a commercial product comprising a mix of short-chain FOS (1-kestose, nystose, and fructosylnystose; FOS) or 10% galacto-oligosaccharides (GOS) (Sigma), and cultivated overnight at 37 °C under anaerobic conditions. This overnight culture was diluted 1 : 10 in fresh MRS broth supplemented with 16% FOS or 10% GOS and cultivated at 37 °C until an $OD_{600\,nm}$ of 0.6–0.7 was reached. Then, bacteria were chilled on ice, harvested by centrifugation (4500 r.p.m. for 15 min), and washed twice with washing buffer composed of 1 mM citrate buffer supplemented with 16% FOS or 10% GOS (pH 6.0). Finally, cells were resuspended in about 1/250 of the original culture volume of ice-cold washing buffer, dispensed in Eppendorf tubes and incubated at 4 °C for 30 min to 3 h.

Electroporation

Plasmid DNA (200 ng) was mixed with 80 μL bacterial suspension in a precooled Gene Pulser disposable cuvette with an interelectrode distance of 0.2 cm (Eppendorf). A high-voltage electric pulse was delivered employing a Gene Pulser apparatus (BioRad, UK) using 25 μF capacity and a parallel resistance of 200 Ω. Following electroporation, bacteria were diluted with 920 μL cMRS broth. Bacteria were incubated for 3 h at 37 °C in an anaerobic cabinet to facilitate cell recovery and expression of the antibiotic resistance marker, after which cells were plated on cMRS agar supplemented with 3 μg mL^{-1} chloramphenicol. Plates were then incubated anaerobically at 37 °C for 48–72 h.

Selection of the transformants

Transformants were cultivated on cMRS supplemented with chloramphenicol at a final concentration of 3 μg mL^{-1}.

DNA was extracted from colonies using GeneReleaser (BioVentures), and the presence of pNZ8048 in transformants was confirmed by PCR using the primers pNZFw (5′-TTTGCAGCGAAGATGTTGTC-3′) and pNZRv (5′-CTATAGCTAACGCCGCAACC-3′) targeting DNA regions on this plasmid. The transformation efficiency was calculated according to the following formula:

$$E = \frac{[\sum c \times (1 \times 10^{x})]}{(\text{DNA } \mu\text{g})}$$

where Σ c is the total number of transformants and x is the dilution factor applied.

Transformation experiments were performed in triplicate.

Plasmid stability studies

Transformants were inoculated into fresh broth in the presence of chloramphenicol and grown for 24 h. These cultures were then screened for plasmid content prior to the start of the experiment to ensure that plasmid pNZ8048 was present. Cultures were then diluted (1%) in fresh broth without chloramphenicol, followed by continuous subcultivation for 15 days by dilution into fresh broth every 24 h in the absence of antibiotic selection. To determine plasmid stability, at least 50 colonies from each tested transformant were transferred to cMRS agar plates with or without chloramphenicol (3 μg mL^{-1}). Growth of these colonies was monitored following 24 h of incubation, and plasmid extractions were performed where relevant.

Mouse colonization

All animals used in this study were cared for in compliance with guidelines established by the Italian Ministry of Health. All procedures were approved by the University of Parma, as executed by the Institutional Animal Care and Use Committee (Dipartimento per la Sanità Pubblica Veterinaria, la Nutrizione e la Sicurezza degli Alimenti Direzione Generale della Sanità Animale e del Farmaco Veterinario). Two groups, each containing six animals of 3-month-old female BALB/c mice, were orally inoculated with bacteria or with water. Bacterial colonization was established by five consecutive daily administrations whereby each animal received 20 μL of 10^9 mL^{-1} of cells using a micropipette tip placed immediately behind the incisors (Sleator *et al.*, 2001). Bifidobacterial inocula were prepared by growing *B. bifidum* PRL2010 containing pNZ8048 anaerobically overnight at 37 °C in cMRS broth containing 3 μg mL^{-1} chloramphenicol. Cultures were harvested by centrifugation (950 ***g*** for 8 min), washed, and resuspended in 100 μL of water. The viable count of each inoculum was determined by retrospective plating on cMRS containing the antibiotic. To estimate the number of *B. bifidum* PRL2010 cells per gram of feces, individual fecal samples were weighed and followed by serial dilution and culturing on selective cMRS agar with chloramphenicol. Following enumeration of *B. bifidum* PRL2010 in fecal samples, 100 random colonies were further tested to verify their identity by the use of PCR primers targeting the *pil*2 and *pil*3 loci (Foroni *et al.*, 2011).

Results and discussion

It has previously been reported that *B. bifidum* cells are practically nontransformable (Argnani *et al.*, 1996). To corroborate such findings, we employed a previously described transformation protocol for *B. bifidum* PRL 2010 (Turroni *et al.*, 2010) and *B. asteroides* PRL2011 (F. Bottacini, F. Turroni, and M. Ventura, unpublished data), which is highly effective for other bifidobacterial strains, such as *Bifidobacterium breve* UCC2003 (O'Connell Motherway *et al.*, 2009). However, as displayed in Table 2, no PRL2010 transformants were obtained using this procedure. Thus, to genetically access *B. bifidum* PRL2010 and *B. asteroides* PRL2011, for which the genome sequences are currently available (F. Bottacini, F. Turroni, and M. Ventura, unpublished data), an efficient transformation protocol is required. Accordingly, we assessed and varied various critical parameters of the bacterial transformation protocol, such as preparation of

Table 2. Effect of various parameters on *Bifidobacterium bifidum* PRL2010 electroporation rates.

Parameters			Rate of transformation (CFU per μg DNA)
Carbohydrate		Growth phase	
Modified Rogosa Medium (O'Connell Motherway *et al.*, 2009)		OD value of 0.6–0.7	0
FOS		OD value of 0.4	0
		OD value of 0.7	1.3×10^3
GOS		OD value of 0.4	0
		OD value of 0.7	3.7×10^3
Resistances		Voltages (kV cm^{-1})	
FOS	100 Ω	7.5	0
		12.5	0
GOS		7.5	0
		12.5	0
FOS	200 Ω	7.5	2.0×10^2
		12.5	1.3×10^3
GOS		7.5	4.1×10^2
		12.5	3.7×10^3

electro-competent cells, electroporation buffers, and electroporation conditions, which are discussed below. Furthermore, susceptibility to the antibiotic used to select transformants (chloramphenicol) was tested for both *B. bifidum* PRL2010 and *B. asteroides* PRL2011 using the MIC assays, which showed a resistance level below 0.5 μg mL^{-1}.

Preparation of electro-competent cells

The presence of a thick and multilayered cell wall in bacteria generally represents a barrier for the uptake of exogenous DNA molecules (Kullen & Klaenhammer, 2000). Bifidobacteria possess a very thick and complex cell wall (Fischer *et al.*, 1987). In particular, for the *B. bifidum* taxon, the peptidoglycan structure differs from that of other bifidobacteria by the existence of specific cross-linking dipeptide bond between the 5-amino group of ornithine and the carboxyl group of C-terminal D-alanine (Veerkamp & van Schaik, 1974). Thus, we attempted to adapt our methodology so as to overcome this physical barrier by varying several parameters such as (1) cultivation of bifidobacteria/transformants in the presence of high concentration of complex carbohydrates; (2) the use of bacterial cells collected at the exponentially growth phase; (3) osmotic stabilizers in washing and electroporation buffers; and (4) maintenance of cells at low temperatures during all steps of the transformation procedure.

Growth media

The addition of carbohydrates at high concentration to the growth medium is a strategy previously described to be effective for transformation of other bifidobacterial species such as *Bifidobacterium animalis, Bifidobacterium longum* subsp. *infantis,* and *Bifidobacterium longum* subsp. *longum* (Argnani *et al.*, 1996; Rossi *et al.*, 1996; Guglielmetti *et al.*, 2007, 2008). In fact, the presence of a high concentration of carbohydrates in the growth medium and in the electroporation buffer has proven to be essential, as no transformants were observed when bacteria were cultivated in the absence of an osmotic stabilizer (Argnani *et al.*, 1996). A similar strategy was followed also for the preparation of the electro-competent *B. bifidum* PRL2010 cells, which were cultivated in the presence of different complex carbohydrates such as FOS or GOS.

Interestingly, PRL2010 transformants were isolated when cells were grown in MRS supplemented with FOS at a final content of 16% as well as with MRS enriched by 10% GOS with a transformation efficiency of 10^3 CFU μg^{-1} DNA (Table 2). Such findings may be explained by the effects that these oligosaccharides have on the composition of the cell wall as well as on other cell envelope constituents (e.g. decreased thickness of capsular polysaccharide layers and/or reduction of the cell wall/capsular complexity). Furthermore, the presence of a high amount of complex carbohydrates in the growth medium may exert a protective action against the stressful conditions encountered by bifidobacterial cells during transformation (Guglielmetti *et al.*, 2008).

Growth phase

Previous studies have reported that the composition of the bacterial cell wall, and consequently the efficiency of DNA uptake, seems to be significantly influenced by the growth phase of the bacterial cells (Rossi *et al.*, 1996). Thus, based on the growth curve of *B. bifidum* PRL2010 cells cultivated on MRS, we harvested PRL2010 cells at different time points corresponding to early (OD value of 0.4) and late exponential phase (OD value of 0.7) (Fig. 1). Subsequently, such cells were submitted to the electroporation procedure, and corresponding transformation efficiency was evaluated (Table 2). Notably, the maximal transformation efficiency was observed when PRL2010 cells were collected at late log phase (Table 2).

Electroporation buffers

Incubation of the cells in an electroporation buffer was found to be crucial for *Bifidobacterium* transformation (Argnani *et al.*, 1996). We observed that storage of bacterial cells for two hours before electroporation at 4 °C in an electroporation buffer composed of 16% FOS or 10% GOS and 1 mM citrate buffer (pH 6.0) significantly improved their transformation efficiency, increasing from $< 10^2$ to 10^4 CFU per μg DNA. Under these conditions, we assume that the low molarity of ammonium citrate acts as an osmotic stabilizer that supports controlled cell envelope removal/degradation without affecting cell viability, which may then result in improved cell wall permeability for exogenous DNA.

Electroporation condition and identification of PRL2010 transformants

Resistances of 100 or 200 Ω and voltages between 7.5 and 12.5 kV cm^{-1} were tested. Optimal results were obtained when the voltage applied to the cuvette was 12.5 kV cm^{-1} and the resistance was set at 200 Ω. When the resistance was set at 100 Ω, no transformants was observed. The transformation efficiency achieved with a voltage of 7.5 kV cm^{-1} and a resistance of 200 Ω was low (Table 2). After incubation, the transformants were selected on MRS supplemented with chloramphenicol and incubated at 37 °C. The presumptive transformants were

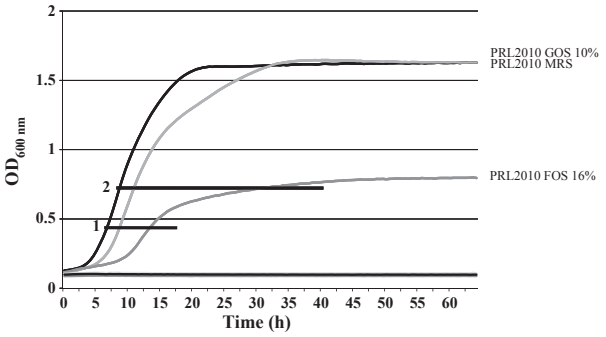

Fig. 1. Growth curves of *Bifidobacterium bifidum* PRL2010 (10%) in a growth medium containing different carbohydrates as carbon sources. Times of cell collection corresponded to early (OD value of 0.4) and late exponential phase (OD value of 0.7).

verified by colony PCR using primers based on the DNA sequence of pNZ8048. The transformation efficiency was calculated to be 1.35×10^3 CFU per μg DNA when the strain was grown in FOS, and 3.7×10^3 CFU per μg DNA when grown in GOS (Table 2).

Plasmid stability was evaluated by continuous cultivation for 15 days of five PRL2010 transformants in the absence of chloramphenicol selection by PCR assays. Notably, all PRL2010 transformants tested did not exhibit any plasmid loss during this period, despite the absence of antibiotic selection.

Transformation of *B. asteroides*

To evaluate the general usefulness of the transformation protocol developed here, we decided to apply it to another *Bifidobacterium* species, *B. asteroides* PRL2011, whose genome was recently decoded (F. Bottacini, F. Turroni and M. Ventura, unpublished data). Interestingly, the *B. asteroides* species represents a distantly related taxon with respect to *B. bifidum*, while it also occupies a different ecological niche, that is, the hindgut of honeybee (Veerkamp & van Schaik, 1974; Fischer *et al.*, 1987; Argnani *et al.*, 1996; de Ruyter *et al.*, 1996; Hartke *et al.*, 1996; Rossi *et al.*, 1996; Kullen & Klaenhammer, 2000; Sleator *et al.*, 2001; Schell *et al.*, 2002; Ventura *et al.*, 2006, 2007, 2009; Guglielmetti *et al.*, 2007, 2008; Sela *et al.*, 2008; O'Connell Motherway *et al.*, 2009; Turroni *et al.*, 2010, 2011; Foroni *et al.*, 2011; Serafini *et al.*, 2011). Thus, one may argue that the *B. asteroides* species possesses a different cell envelope composition (e.g. exopolysaccharides, extracellular proteins) compared to that of *B. bifidum*. When the transformation protocol optimized on *B. bifidum* PRL2010 cells was employed for transforming *B. asteroides* PRL2011 using pNZ8048, a higher transformation efficiency (1.6×10^4 CFU per μg DNA) was obtained as compared to *B. bifidum* PRL2010.

Bifidobacterium bifidum PRL2010 in colonization *in vivo* experiments

A direct application from the results of the successful transformation protocol described in this study was to monitor the colonization efficiency of *B. bifidum* PRL2010 in a murine model. In fact, so far, it has been proven impossible to generate stable antibiotic-resistant *B. bifidum* PRL2010 derivatives by spontaneous mutation such as those in other bacterial species might be obtained upon repeated cultivation in the presence of antibiotics. Thus, to discriminate the presence of PRL2010 cells from other members of the gut microbiota of mice, we employed a derivative PRL2010 strain that contained a plasmid carrying an antibiotic resistance gene to act as a selective marker.

The normal microbiota of mice encompasses microorganisms that are sensitive to chloramphenicol (Savino *et al.*, 2011), thus indicating that this antibiotic can be used in selective media. Colonization and clearance of PRL2010 were monitored over a 15-day period by determining viable counts recovered from fecal samples. Two groups of six mice were fed orally on a daily basis with either PRL2010 containing pNZ8048 (designated here as $PRL2010_{pNZ8048}$) or water for 1 week. In addition, 5% (w/v) FOS was orally administered to the mice, in water suspension, throughout the experiment to further facilitate *Bifidobacterium* colonization. After 1 week, the $PRL2010_{pNZ8048}$ supplementation was discontinued, and after one additional week, the animals were killed.

To follow $PRL2010_{pNZ8048}$ colonization, fecal samples were collected periodically (on days 0, 2, 5, 9, 12, and 15), and $PRL2010_{pNZ8048}$ cell enumeration was performed by plating fecal material on MRS–Cys–Agar supplemented with chloramphenicol. After incubation at 37 °C, the identity of colonies grown on MRS supplemented with chloramphenicol was further evaluated using PCR and employing PRL2010-specific primers that target pili-encoding loci, which have been described previously (Turroni *et al.*, 2010; Foroni *et al.*, 2011). The inoculated bacterial population increased in number (Fig. 2), reaching a maximum of 10^7 CFU g^{-1} feces at day 5.

Interestingly, following this rapid increase of PRL2010 cell numbers during the period of bacterial supplementation, the level of PRL2010 cells decreased to reach a plateau of approximately 10^5 CFU that appeared to remain stable during the full length of the post-treatment period (Fig. 2). Notably, the presence of high numbers of $PRL2010_{pNZ8048}$ cells upon a period of 7 days without any supplementation with bifidobacterial cells reinforces the notion that the plasmid is stable. Altogether these data indicate that PRL2010 is capable of colonizing the intestine of mouse, which will open new avenues in the

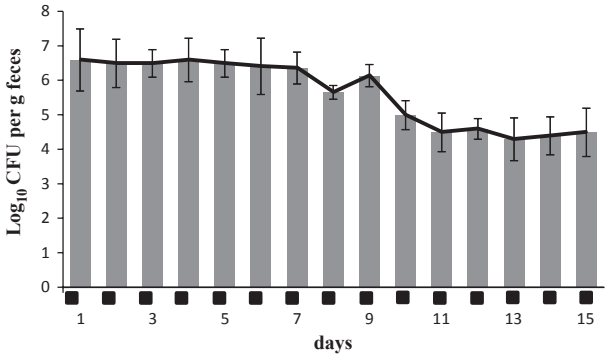

Fig. 2. Population sizes of *Bifidobacterium bifidum* PRL2010 colonizing the intestine of BALB/c mice. Each point represents the average of the log-transformed population size ± standard deviation for six mice. Squares represent the population size of PRL2010 in control mice.

exploration of host–microbe interactions of this microorganism using an *in vivo* murine model (O'Connell Motherway *et al.*, 2011).

Conclusion

This study describes an optimized protocol for the transformation of bifidobacteria that enables the establishment of plasmid DNA into two very distantly related species, that is, *B. bifidum* and *B. asteroides* taxa, where in the latter case it represents the first report on plasmid-mediated transformability. The transformation rates achieved were sufficiently high for cloning purposes; nonetheless, the experiments so far performed highlighted transformation efficiency of 10^4 CFU μg^{-1} which is not yet high enough for site-directed mutagenesis and for an effective selection of transformants in gene knock-out experiments (O'Connell Motherway *et al.*, 2009). The next step will be to improve the transformation efficiency, which could be achieved by overcoming the restriction modification systems of this microorganism (O'Connell Motherway *et al.*, 2009). Genetic tools to manipulate bifidobacteria are still largely undeveloped and represent a bottleneck in the advancing of knowledge on this important group of microorganisms. Thus, the transformation protocol and subsequent colonization model described in this study offer two important adjuncts in exploring genomic functionalities of bifidobacteria under *in vitro* as well as *in vivo* conditions.

Acknowledgements

We thank GenProbio srl for the financial support of the Laboratory of Probiogenomics. This work was financially supported by a FEMS Advanced Fellowship 2011 and an IRCSET Embark postdoctoral fellowship to F.T. The Alimentary Pharmabiotic Centre, which is a Centre for Science and Technology (CSET), is funded by Science Foundation Ireland (SFI), through the Irish Government's National Development Plan (Grant no. 02/CE/B124 and 07/CE/B1368).

References

Argnani A, Leer RJ, van Luijk N & Pouwels PH (1996) A convenient and reproducible method to genetically transform bacteria of the genus *Bifidobacterium*. *Microbiology* **142**(Pt 1): 109–114.

de Ruyter PG, Kuipers OP & de Vos WM (1996) Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin. *Appl Environ Microbiol* **62**: 3662–3667.

Fischer W, Bauer W & Feigel M (1987) Analysis of the lipoteichoic-acid-like macroamphiphile from *Bifidobacterium bifidum* subspecies pennsylvanicum by one- and two-dimensional 1H- and 13C-NMR spectroscopy. *Eur J Biochem* **165**: 647–652.

Foroni E, Serafini F, Amidani D *et al.* (2011) Genetic analysis and morphological identification of pilus-like structures in members of the genus *Bifidobacterium*. *Microb Cell Fact* **10** (Suppl 1): S16.

Guglielmetti S, Karp M, Mora D, Tamagnini I & Parini C (2007) Molecular characterization of *Bifidobacterium longum* biovar *longum* NAL8 plasmids and construction of a novel replicon screening system. *Appl Microbiol Biotechnol* **74**: 1053–1061.

Guglielmetti S, Ciranna A, Mora D, Parini C & Karp M (2008) Construction, characterization and exemplificative application of bioluminescent *Bifidobacterium longum* biovar *longum*. *Int J Food Microbiol* **124**: 285–290.

Hartke A, Benachour A, Boutibonnes P & Auffray Y (1996) Characterization of a complex restriction/modification system detected in a *Bifidobacterium longum* strain (vol 45, pg 132, 1995). *Appl Microbiol Biotechnol* **46**: 202.

Kullen MJ & Klaenhammer TR (2000) Genetic modification of intestinal lactobacilli and bifidobacteria. *Curr Issues Mol Biol* **2**: 41–50.

O'Connell Motherway M, O'Driscoll J, Fitzgerald GF & Van Sinderen D (2009) Overcoming the restriction barrier to plasmid transformation and targeted mutagenesis in *Bifidobacterium breve* UCC2003. *Microb Biotechnol* **2**: 321–332.

O'Connell Motherway M, Zomer A, Leahy SC *et al.* (2011) Functional genome analysis of *Bifidobacterium breve* UCC2003 reveals type IVb tight adherence (Tad) pili as an essential and conserved host-colonization factor. *P Natl Acad Sci USA* **108**: 11217–11222.

Rossi M, Brigidi P, Gonzalez Vara y Rodriguez A & Matteuzzi D (1996) Characterization of the plasmid pMB1 from *Bifidobacterium longum* and its use for shuttle vector construction. *Res Microbiol* **147**: 133–143.

Savino F, Roana J, Mandras N, Tarasco V, Locatelli E & Tullio V (2011) Faecal microbiota in breast-fed infants after antibiotic therapy. *Acta Paediatr* **100**: 75–78.

Schell MA, Karmirantzou M, Snel B *et al.* (2002) The genome sequence of *Bifidobacterium longum* reflects its adaptation to the human gastrointestinal tract. *P Natl Acad Sci USA* **99**: 14422–14427.

Sela DA, Chapman J, Adeuya A *et al.* (2008) The genome sequence of *Bifidobacterium longum* subsp. *infantis* reveals adaptations for milk utilization within the infant microbiome. *P Natl Acad Sci USA* **105**: 18964–18969.

Serafini F, Bottacini F, Viappiani A, Baruffini E, Turroni F, Foroni E, Lodi T, van Sinderen D & Ventura M (2011) Insights into physiological and genetic mupirocin susceptibility in bifidobacteria. *Appl Environ Microbiol* **77**: 3141–3146.

Sleator RD, Gahan CG & Hill C (2001) Mutations in the listerial *pro*B gene leading to proline overproduction: effects on salt tolerance and murine infection. *Appl Environ Microbiol* **67**: 4560–4565.

Turroni F, Bottacini F, Foroni E *et al.* (2010) Genome analysis of *Bifidobacterium bifidum* PRL2010 reveals metabolic pathways for host-derived glycan foraging. *P Natl Acad Sci USA* **107**: 19514–19519.

Turroni F, van Sinderen D & Ventura M (2011) Genomics and ecological overview of the genus *Bifidobacterium*. *Int J Food Microbiol* **149**: 37–44.

Veerkamp JH & van Schaik FW (1974) Biochemical changes in *Bifidobacterium bifidum* var. Pennsylvanicus after cell wall inhibition. VII. Structure of the phosphogalactolipids. *Biochim Biophys Acta* **348**: 370–387.

Ventura M, Canchaya C, Del Casale A, Dellaglio F, Neviani E, Fitzgerald GF & van Sinderen D (2006) Analysis of bifidobacterial evolution using a multilocus approach. *Int J Syst Evol Microbiol* **56**: 2783–2792.

Ventura M, Canchaya C, Tauch A, Chandra G, Fitzgerald GF, Chater KF & van Sinderen D (2007) Genomics of Actinobacteria: tracing the evolutionary history of an ancient phylum. *Microbiol Mol Biol Rev* **71**: 495–548.

Ventura M, O'Flaherty S, Claesson MJ, Turroni F, Klaenhammer TR, van Sinderen D & O'Toole PW (2009) Genome-scale analyses of health-promoting bacteria: probiogenomics. *Nat Rev Microbiol* **7**: 61–71.