Developing an efficient and reproducible conjugation-based gene transfer system for bifidobacteria

Wilfredo Dominguez and Daniel J. O’Sullivan

Microbiology (2013), 159, 328–338
DOI 10.1099/mic.0.061408-0

INTRODUCTION

Bifidobacteria are widely used as probiotics and have attracted increasing research interest worldwide. However, molecular techniques are still very scarce mainly due to the low efficiencies and strain-specific electroporation protocols that have been developed. Bacterial conjugation enables the transfer of genetic material among a relatively wide range of organisms and with virtually no size limitation. A conjugation protocol was developed based on the RP4 conjugative machinery in the Escherichia coli strain WM3064(pBB109). Using this machinery, the newly constructed transmissible E. coli–Bifidobacterium shuttle vector, pDOJHR-WD2, was successfully and consistently transferred into several strains representing four Bifidobacterium species at efficiencies which correlated with the E. coli to bifidobacteria ratios. Higher ratios were found to significantly improve transfer frequency per recipient, with almost 100% transfer frequency occurring when the ratio was 10^5 : 1. The incompatible resident plasmid, pDOJH10S, in Bifidobacterium longum DJO10A was able to coexist, albeit at lower copy numbers, with the incoming vector pDOJHR-WD2 even though they possess the same ori. In some cases the copy number of this resident plasmid was too low to observe via gel electrophoresis, but it could be detected by Southern hybridization. Plasmid curing resulted in a strain, DJO10A-W3, that had lost both plasmids and this showed a one-log increase in conjugation efficiency due to the lack of plasmid incompatibility. In conclusion, this novel conjugative gene transfer protocol can be used for the introduction of genetic material (without size restriction) into Bifidobacterium species and is particularly useful for strains that are recalcitrant to electroporation.

Bacterial conjugation is a genetic exchange mechanism that requires direct contact between cells and was first proposed to describe DNA transfer between different strains of Escherichia coli (Lederberg & Tatum, 1946). Today, conjugation techniques have been used in virtually all cultured Gram-negative bacteria and have been reported in several Gram-positive bacteria (Reviewed by Schroeder & Lanka, 2005). However, the only report on conjugative gene transfer in the Bifidobacterium genus reported that it was unsuccessful (Shkporov et al., 2008).

The bacterial conjugation machinery is composed of an oriT sequence and tra genes (reviewed by Smillie et al., 2010). The oriT sequence is the only component needed in cis to transfer a plasmid through conjugation, providing that the Tra functions are present either in cis or in trans. The tra genes encode a relaxase, a mating pair formation (MPF) complex and a type IV coupling protein. The oriT is recognized by the relaxase, which binds and nicks the DNA at the nic site within the oriT (Varsaki et al., 2009). The relaxase together with one or several auxiliary proteins are termed the relaxosome (Pansegrau et al., 1990). The

Abbreviations: DAP, diaminopimelic acid; MPF, mating pair formation; R/M, restriction–modification.

A set of supplementary methods, a supplementary figure and two supplementary tables are available with the online version of this paper.

Correspondence
Daniel J. O’Sullivan
dosulliv@umn.edu

Received 15 June 2012
Revised 2 November 2012
Accepted 27 November 2012
relaxosome generates a ssDNA strand that is transferred into the recipient cell. The type IV coupling system serves as a bridge between the relaxosome and the MPF, a type IV secretion system (Mihajlovic et al., 2009). This secretion system transfers the ssDNA-bound protein complex into the recipient cell (Cascales & Christie, 2004). In addition, the secretion system mediates the initial cell-to-cell contact through a specialized structure, termed a pilus, which is depolymerized to bring the mating cells together (Smillie et al., 2010). It has been proposed that the characteristics of a particular pilus dictate, in part, the ability of the conjugative system to mate with different cells and under different environmental conditions (Bradley, 1984).

The naturally occurring conjugative plasmids have been classified into four groups represented by the F, R64, ICEHIN1056 and pTi plasmids as prototypes (reviewed by Smillie et al., 2010). The pTi plasmid, together with the RP4 and other plasmids, belongs to the incompatibility group P (IncP) and are known for their ability to replicate and conduct conjugal transfer among a wide range of bacteria (reviewed by Thomas & Smith, 1987). The RP4 plasmid is 60 kb and was first described in *Pseudomonas aeruginosa* (Datta et al., 1971). The plasmid contains 74 ORFs (Pansegrau et al., 1994) and allows efficient mating only on solid surfaces (Bradley, 1980, 1984). While RP4 only replicates in Gram-negative bacteria, its Tra functions have been used to promote conjugation between *E. coli* and a diverse range of bacteria, as well as yeast and mammalian cells, demonstrating its ability to form functional pili between various organisms (Bates et al., 1998; Waters, 2001). The RP4 Tra functions have been used successfully to transfer plasmids from *E. coli* to several *Actinobacteria*, including members of the genera *Corynebacterium* (Schäfer et al., 1994), *Streptomyces* (Luzhetskyy et al., 2006), *Rhodococcus* (Desomer et al., 1988) and *Mycobacterium* (Gormley & Davies, 1991).

As discussed above, electroporation in *Bifidobacterium* species has proven to be challenging. It has been proposed that this difficulty is mainly due to restriction–modification (R/M) systems in the different strains that efficiently degrade foreign DNA (Kim et al., 2010; O’Connell Motherway et al., 2009; Yasui et al., 2009). This hypothesis is supported by the observation that plasmid DNA isolated from a *Bifidobacterium* transformant exhibited higher electroporation efficiencies than plasmid DNA isolated from an *E. coli* host (Rossi et al., 1998; Shkoporov et al., 2008; Yasui et al., 2009). This limitation has been overcome for specific R/M systems by cloning the cognate methylase gene in *E. coli* (Yasui et al., 2009) and by in vitro methylation (Kim et al., 2010; O’Connell Motherway et al., 2009). However, these methodologies require strain-specific designs and knowledge of the resident R/M systems.

In our laboratory, exhaustive attempts using published and modified electroporation protocols were conducted to introduce a shuttle vector into different *Bifidobacterium longum* and *Bifidobacterium animalis* subsp. *lactis* strains. Very low transfer efficiency was achieved only with some strains and it was not reliable, requiring many attempts for each. This prompted us to develop a functional conjugative system to introduce the vector into bifidobacteria, as conjugation has at least two important advantages over electroporation. First, electroporation efficiencies are known to decrease as the size of the vector increases (Sjostková & Horákova, 1998). Conjugation does not have this limitation and has been successfully used to transfer entire genomes in *E. coli* (Isacs et al., 2011). Second, during conjugation the DNA is transferred from the donor to the recipient cell as a single strand which is methylated following second strand synthesis, thus bypassing the restriction systems of the cells. Therefore, the objective of this study was to develop the first functional conjugation system that could enable intergenic transfer of genetic material between *E. coli* and *Bifidobacterium* species using the RP4 machinery.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** The *Bifidobacterium* and *E. coli* strains used in this study are listed in Table 1. Bifidobacteria were grown anaerobically at 37 °C in de Man, Rogosa and Sharpe (MRS) medium (Difco) supplemented with 0.05% l-cysteine HCl (Sigma–Aldrich). When needed for selection, 2.5 µg chloramphenicol ml⁻¹ was added to the media for all bifidobacteria except for *B. animalis subsp. lactis* BD-12, which required 6 µg ml⁻¹. For erythromycin selection, 2 µg ml⁻¹ was used. *E. coli* WM3064 was grown at 37 °C with shaking at 250 r.p.m. in Brain Heart Infusion (BHI) medium (Difco) and 25 µg dianisopimelic acid (DAP) ml⁻¹; when needed, this was supplemented with 20 µg chloramphenicol ml⁻¹ and 50 µg kanamycin ml⁻¹. *E. coli* DH5α was grown at 37 °C with shaking at 250 r.p.m. in LB broth when selection with 20 µg chloramphenicol ml⁻¹ was required or in BHI broth when selection with 175 µg erythromycin ml⁻¹ was used. Conjugations were conducted on Bifidobacteria Low Iron Medium with Iron (BLIM + Fe) (Islam, 2006) agar plates containing 2.0% proteose peptone, 0.15% K₂HPO₄, 0.5% glucose, 0.15% MgSO₄ . 7H₂O, 0.05 mM FeCl₃, 100 mM PIPES (MP Biomedicals) and 1.8% agar with the pH adjusted to 7.0 with 9 M NaOH. Growth was routinely monitored by measuring OD₆₀₀ using a Spectronic 20D spectrophotometer (Milton Roy Company).

**Molecular techniques.** Total DNA from bifidobacteria was isolated by pelleting the cells from a 15 ml fully grown culture, washing and resuspending in 450 µl lysozyme solution (100 mg ml⁻¹). The mixture was incubated at 37 °C for 1 h, placed at −80 °C for 10 min and incubated at 37 °C for 10 min. The cells were then mixed with 200 µl protease K solution (Qiagen) and incubated at 37 °C for 1 h. The mixture was centrifuged and the supernatant was extracted with an equal volume of phenol/chloroform/isooamyl alcohol (25:24:1) saturated with 10 mM Tris, pH 8.0, 1 mM EDTA (Sigma–Aldrich). The upper layer was transferred to a new tube where the residual RNA was digested with 3 µl RNase A (100 mg ml⁻¹) for 1 h at 37 °C. A second extraction was conducted with an equal volume of phenol/chloroform/isooamyl alcohol. The upper layer was transferred to a new tube and the DNA was precipitated with an equal volume of 2-propanol and 1/10th volume of 3 M potassium acetate (pH 5.5). The DNA was collected by centrifugation (16 000 × g, 4 °C, 20 min) and resuspended by incubating at 55 °C for 1 h in molecular-grade water.
Conjugation. An optimized protocol for intrageneric plasmid transfer between *E. coli* and *bifidobacteria* consisted of the following. Fully grown cultures of *bifidobacteria* strains were diluted 1:15 into fresh media and incubated at 37 °C anaerobically until they reached OD_{600} 0.8–0.9. The cells were harvested by centrifugation (2000 g, 25 °C and 5 min) and washed with 1/15th culture volume of fresh BHI broth and resuspended in approximately 1/20th of the culture volume. The donor *E. coli* strain was diluted 1:20 into fresh medium from an overnight culture and incubated at 37 °C with shaking at 250 r.p.m. to OD_{600} 0.6. The cells were harvested by centrifugation (2000 g, 25 °C and 5 min) and washed with 1/50th of the culture volume using fresh BHI broth. The donor and recipient strains were mixed by brief vortexing. The mixture was collected by centrifugation (16,100 g, 25 °C and 30 s) and resuspended in 100 μl fresh BHI broth. The cells were then spotted on BLIM + Fe and incubated aerobically at 37 °C for 12 h. The cells were collected using a loop and resuspended in 1 ml MRS supplemented with 0.05% cysteine and 10% glycerol (v/v). An aliquot of the suspension was plated on MRS agar supplemented with 0.05% cysteine and chloramphenicol. The plates were incubated for 2–3 days anaerobically.

Plasmid curing. Plasmid curing was conducted on *B. longum* DJO10A transconjugants. The strain was inoculated into MRS broth supplemented with 4 μg ethidium bromide ml⁻¹. The culture was incubated anaerobically at 37 °C for 24 h. Single colonies were obtained by streaking the culture on MRS agar and incubating at 37 °C anaerobically. The plasmid-cured colonies were screened by patch-plating individual colonies on duplicate MRS plates with and without chloramphenicol. Plasmid profiles analysis was conducted on chloramphenicol-sensitive colonies.
Southern hybridization. Approximately 20 μg total DNA per sample was digested with BamHI and electrophoresed through a 0.9 % agarose gel in TAE buffer at 5 V cm⁻¹. The gel was then prepared by denaturing the DNA with an alkaline treatment and transferred to a positively charged nylon membrane (Roche) using capillary transfer. The DNA was fixed to the membrane using the auto-cross-link function of a UV Stratalinker 2400 cross-linker (Stratagene). The gene images AlkPhos direct labelling and detection system with CDP-Star (GE Healthcare) was used for labelling, hybridization and detection according to the manufacturer’s protocols. The probes were labelled using DNA obtained from gel-extracted pDOJH10S and the PCR products of GroE-F and GroE-R. The entire 1 kb ladder was used as probe and separately hybridized to the fragment of the membrane that contained it.

Dot blot hybridization. Approximately 6 μg DNA per spot was denatured using an alkaline treatment and then transferred to a positively charged nylon membrane (Roche) using the Bio-Dot apparatus (Bio-Rad) following the manufacturer’s recommendations. The DNA was fixed to the membrane and hybridized as described above for the Southern hybridization.

RESULTS

Electroporation into bifidobacteria

Numerous electroporation attempts were conducted using vectors from two incompatibility groups and either chloramphenicol or erythromycin selection marker. Multiple strategies were investigated and tested at least five times independently and these were incubated on selective media for at least 5 days before being categorized as unsuccessful. The protocols employed and results are described in the Supplementary Material (available with the online version of this paper). As electroporation of plasmid DNA isolated from E. coli only resulted in a single transformant of one strain (B. animalis subsp. lactis Bb-12) and B. longum VMK44 was only reproducibly transformed with a vector reisolated from itself, it was concluded that current electroporation protocols were not feasible for reproducible gene transfer into bifidobacteria in general. Therefore, a conjugation approach was investigated.

Construction of a mobilizable shuttle vector for bifidobacteria

The mobilizable plasmid pDOJHR-WD2 (Fig. 1) was constructed by engineering the oriT from pRK2013 into the pDOJHR-WD1 vector. The oriT was amplified using SalI site-flanking primers (Orit-F and Orit-R) and ligated into the XhoI site of pDOJHR-WD1, thus forming TaqI sites at either side of oriT. Therefore, pDOJHR-WD2 is an E. coli–Bifidobacterium shuttle vector with two Gram-positive selection markers and an oriT that can be mobilized by the RP4 conjugative machinery in trans.

Intergeneric conjugation of pDOJHR-WD2 between E. coli and bifidobacteria

The E. coli strain WM3064 (pBB109) was transformed with pDOJHR-WD2. The pBB109 vector in the E. coli strain provided the relaxase functions in trans, which catalyses the nicking of the pDOJHR-WD2 at the oriT sequence. The Tra functions, provided by the chromosomally integrated RP4 in this E. coli strain, form the MPF and the type IV coupling system. The conjugation protocol was optimized by testing different conjugation temperatures (25, 30, 37 or 42 °C) under both aerobic and anaerobic conditions using B. animalis subsp. lactis Bb-12 and B. longum DJO10A. Similar conjugation efficiencies were observed when the

Table 2. Primers used in this study

Restriction enzyme sites are indicated in bold underlined type.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5'-3')</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat-F</td>
<td>TCGCGTATGTGAGGCATTT</td>
<td>60</td>
</tr>
<tr>
<td>Cat-R</td>
<td>AAGAGCGGTATATCGTAGCTT</td>
<td>60</td>
</tr>
<tr>
<td>GroE-F</td>
<td>ACAAACATCGGGACACCTT</td>
<td>54</td>
</tr>
<tr>
<td>GroE-R</td>
<td>AGCTTCAGGCCCAGTTCGT</td>
<td>54</td>
</tr>
<tr>
<td>Orit-F</td>
<td>AGAGAGACAGACGGGAGCCGCGT</td>
<td>65</td>
</tr>
<tr>
<td>Orit-R</td>
<td>AGAGAGAACCAGACGCTT</td>
<td>65</td>
</tr>
<tr>
<td>Plas-F</td>
<td>AATGCATCTTTCCTGTCC</td>
<td>65</td>
</tr>
<tr>
<td>Plas-R</td>
<td>AAACGAGAAGCAACGAGG</td>
<td>65</td>
</tr>
<tr>
<td>Plas1-F</td>
<td>CCGCGTGGCACGCAACAGACT</td>
<td>65</td>
</tr>
<tr>
<td>Plas1-R</td>
<td>CGGCAAGGAGGACGCTT</td>
<td>65</td>
</tr>
<tr>
<td>Plas2-F</td>
<td>ATCACCGCTCAGAAGCAGG</td>
<td>65</td>
</tr>
<tr>
<td>Plas2-R</td>
<td>GGAAGACATCGGACACGCGG</td>
<td>65</td>
</tr>
<tr>
<td>Plas3-F</td>
<td>GCCCAAGTGGCAGATTTG</td>
<td>65</td>
</tr>
<tr>
<td>Plas3-R</td>
<td>GACGGAGAGGGGAGACCTA</td>
<td>65</td>
</tr>
<tr>
<td>Plas4-F</td>
<td>GCTGGGCGTGGCGCTT</td>
<td>65</td>
</tr>
<tr>
<td>Plas4-R</td>
<td>CCGCGGTGCGCTTTTTCAC</td>
<td>65</td>
</tr>
</tbody>
</table>

http://mic.sgmjournals.org 331
mating was conducted at 37 and 42 °C aerobically. However, no transconjugants were observed using the other tested conditions.

To evaluate this conjugation protocol for a range of bifidobacteria, approximately 10^7 mid-exponential-phase E. coli cells, washed with antibiotic-free broth, were used in each conjugation with bifidobacteria. The bifidobacteria cultures were harvested at OD600 0.8–0.9, washed and resuspended in 1/15th of the original volume with fresh broth to remove acids that could inhibit E. coli completely. A defined volume of cells, corresponding to approximately 10^5–10^9 cells depending on the strain, was used in each conjugation (Table 3). Chloramphenicol-resistant transconjugants were counted and sampled to verify the transfer of the vector by conducting plasmid isolations. The conjugation was successful for all the strains tested as assessed by plasmid analysis (Figs 2 and 3a). At least 16 colonies were screened for each strain and no false positives were detected. The efficiencies observed varied from 10^{-4} to 10^{-6} transconjugants per recipient cell (Table 3). It was evident that the higher efficiencies occurred when the ratio of bifidobacteria to E. coli in the conjugation favoured E. coli. The highest transfer efficiency occurred for B. animalis subsp. lactis Bb-12 and that conjugation also had a bifidobacteria to E. coli ratio that favoured E. coli (1:730). To investigate the effect of the ratio, several conjugations were set up between E. coli and B. animalis subsp. lactis Bb-12 with variable ratios of donor and recipient. The results showed a clear and exponential relationship between the donor:recipient ratio and the conjugation efficiency. Ratios favouring E. coli more than approximately 10^5:1 yielded almost 100% efficiency while ratios favouring bifidobacteria more than approximately 10^2:1 yielded no detectable transconjugants (Fig. 4).

### Incompatibility effects of introduced vector with a resident plasmid in B. longum DJO10A

The pDOJHR-WD2 and the pDOJH10S plasmid, one of the resident plasmids in B. longum DJO10A, have the same plasmid origin of replication for bifidobacteria. In fact, the entire sequence of the pDOJH10S is present in pDOJHR-W2. Therefore, it was expected that conjugation with pDOJHR-WD2 would replace the resident plasmid. Interestingly, the plasmid analysis revealed that the two plasmids coexisted in most of the transconjugants (Fig. 3a, lane 5). In only one case, among more than 50 successful transconjugants screened, the plasmid analysis suggested that the incoming vector had replaced the resident plasmid. This strain was designated B. longum DJO10A-W1 (Fig. 3a, lane 4). However, further analysis of the strain through

### Table 3. Efficiencies of intergeneric conjugations between E. coli and Bifidobacterium strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Count (c.f.u.)</th>
<th>Ratio(^{\dagger})</th>
<th>Colonies(^{\ddagger})</th>
<th>Efficiency(^{\dagger\dagger})</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. animalis subsp. lactis Bb-12</td>
<td>5.3 × 10^6</td>
<td>730 : 1</td>
<td>24 ± 11</td>
<td>1.8 × 10^{-4}</td>
</tr>
<tr>
<td>B. bifidum ATCC 15696</td>
<td>1.8 × 10^5</td>
<td>60 : 1</td>
<td>3 ± 2</td>
<td>6.5 × 10^{-4}</td>
</tr>
<tr>
<td>B. breve ATCC 15701</td>
<td>3.7 × 10^8</td>
<td>1 : 36</td>
<td>27 ± 6</td>
<td>2.9 × 10^{-6}</td>
</tr>
<tr>
<td>B. longum subsp. infantis RECl4</td>
<td>1.2 × 10^7</td>
<td>1 : 1</td>
<td>2 ± 2</td>
<td>7.5 × 10^{-6}</td>
</tr>
<tr>
<td>B. longum DJO6A</td>
<td>1.2 × 10^8</td>
<td>1 : 3</td>
<td>51 ± 14</td>
<td>1.7 × 10^{-5}</td>
</tr>
<tr>
<td>B. longum DJO10A</td>
<td>1.5 × 10^8</td>
<td>33 : 1</td>
<td>6 ± 2</td>
<td>1.6 × 10^{-5}</td>
</tr>
<tr>
<td>B. longum VMK44</td>
<td>1.2 × 10^9</td>
<td>1 : 33</td>
<td>91 ± 21</td>
<td>3.0 × 10^{-6}</td>
</tr>
</tbody>
</table>

\(^{\ast}\) Average of four replicates.

\(^{\dagger}\) Ratio of E. coli: bifidobacteria cells in each conjugation.

\(^{\ddagger}\) Average number of transconjugants in four replicate conjugations plated in duplicate plates; approximately 1/40th of the total conjugation volume was plated.

\(^{\dagger\dagger}\) Average number of transconjugants per recipient from four replicate conjugations plated in duplicate.
PCR, using the Plas-F and Plas-R primers targeting a sequence unique to pDOJH10S disrupted when the vector pDOJHR was constructed, revealed the presence of this plasmid at a low copy number, undetectable by plasmid profiling.

To assess the stability of pDOJH10S in the transconjugants, two conjugated strains, one with visible pDOJH10S and the *B. longum* DJO10A-W1 strain, were grown overnight in different concentrations of chloramphenicol (0, 2.5, 5, 7, 10, 15 and 30 μg ml⁻¹). In addition, the two strains were grown for more than 30 generations using 2.5 μg chloramphenicol ml⁻¹. In all cases the plasmid profile was not altered and no loss of the resident pDOJH10S plasmid was evident.

Curing of pDOJHR-WD2 from *B. longum* DJO10A-W1

*B. longum* DJO10A is of particular interest because it is the only strain of bifidobacteria with a publicly available genome sequence that was obtained prior to extensive laboratory culturing. As it exhibits several unique characteristics that are pertinent to survival in the gut, such as the ability to produce a lantibiotic, its study could lead to the development of an improved probiotic bifidobacterium. It contains two plasmids, pDOJH10L and pDOJH10S; the latter contains the same origin of replication as the vector pDOJHR-WD2 used in this study. Thus, a strain free of the resident pDOJH10S could potentially exhibit higher transformation or transconjugation efficiencies. To investigate if this was the case, *B. longum* DJO10A-W1 was subjected to curing as described in Methods. Plasmid analysis of chloramphenicol-sensitive colonies only revealed the pDOJH10L native plasmid of the parent strain (Fig. 3b). One of the chloramphenicol-sensitive colonies was designated *B. longum* DJO10A-W2. Further analysis of DJO10A-W2 using PCR with the primer pairs Plas1-F/R, Plas3-F/R and Plas4-F/R (Table 2) revealed the presence of either pDOJH10S or pDOJHR-W2. The chloramphenicol-sensitive phenotype and the negative PCR with the primer pairs Plas-F/R and Plas2-F/R suggested that pDOJH10S was present in the strain, possibly integrated in to the chromosome. To further investigate this, a dot blot
hybridization using the entire plasmid pDOJH10S as a probe was conducted. Interestingly, the assay was positive for both DJO10A-W1 and DJO10A-W2 (Fig. 5a). To investigate if the plasmid was present in circular form or inserted in the chromosome, a Southern hybridization of BamHI-digested total chromosomal DNA was conducted using pDOJH10S as a probe. This assay indicated that the strain DJO10A-W1 contained both plasmids in circular form and DJO10A-W2 contained pDOJHR-W2 in circular form. This was surprising as the strain is chloramphenicol-sensitive. The chloramphenicol resistance gene, cat, and its promoter region from DJO10A-W2 were sequenced using the primers Cat-F and Cat-R (Table 2). The sequencing data showed several mutations in the cat gene rendering it non-functional. To investigate if this phenomenon was reproducible, a new curing experiment was conducted. From this experiment, chloramphenicol-sensitive colonies were screened for pDOJH10S and pDOJHR-WD2 using PCR. Interestingly, one other colony contained the pDOJH10S and the other four were free of either plasmid. One of these plasmid-free strains was designated DJO10A-W3.

To assess the influence of the resident plasmid on the conjugation efficiency, conjugations were conducted using the wild-type, DJO10A-W2 and DJO10A-W3 strains. These strains contain high, low and zero copy numbers of the pDOJH10S origin of replication respectively. The influence of the origin of replication is evident as the absence of the plasmid increased the efficiency by nearly a log (Table 4).

![Fig. 4. Relationship between donor (E. coli WD3064)/recipient (B. animalis subsp. lactis Bb-12) ratio and conjugation efficiencies. Detection limit for transconjugants was calculated as 10^{-7}.](image-url)

![Fig. 5. (a) Dot blot hybridization of total DNA from (1) B. longum DJO10A, (2) B. longum DJO10A-W1, (3) B. longum DJO10A-W2 and (4) B. animalis subsp. lactis Bb-12 as a negative control, with probes targeting (i) groEL gene as a positive control and (ii) the whole pDOJH10S plasmid. (b) Southern blot showing (i) 1 kb ladder, (ii) total DNA from B. longum DJO10A-W1 digested with BamH1 and (iii) total DNA from B. longum DJO10A-W2 digested with BamH1 and hybridized with the whole pDOJH10S plasmid and groEL gene fragment as probes. Arrows indicate: 1, groEL in a 22.7 kb BamH1 fragment; 2, pDOJH10L (~10 kb) containing a 708 bp segment with 74% identity to pDOJH10S; 3, pDOJHR-WD2 (8.2 kb) constructed with the entire pDOJH10S plasmid; and 4, pDOJH10S (3.6 kb). All plasmids contain a single BamH1 restriction site.](image-url)
**Table 4. Efficiencies of intergeneric conjugations between E. coli and B. longum DJO10A strains**

<table>
<thead>
<tr>
<th>B. longum strain</th>
<th>Count (c.f.u.)*</th>
<th>Ratio†</th>
<th>Colonies‡</th>
<th>Efficiency§</th>
</tr>
</thead>
<tbody>
<tr>
<td>DJO10A</td>
<td>$1.5 \times 10^9$</td>
<td>33:1</td>
<td>$6 \pm 2$</td>
<td>$1.6 \times 10^{-5}$ a</td>
</tr>
<tr>
<td>DJO10A-W2</td>
<td>$2.4 \times 10^9$</td>
<td>21:1</td>
<td>$28 \pm 6$</td>
<td>$4.7 \times 10^{-5}$ b</td>
</tr>
<tr>
<td>DJO10A-W3</td>
<td>$3.5 \times 10^9$</td>
<td>14:1</td>
<td>$19 \pm 7$</td>
<td>$2.2 \times 10^{-4}$ c</td>
</tr>
</tbody>
</table>

*Average of four replicates. †Ratio of E. coli:bifidobacteria cells in each conjugation. ‡Average number of transconjugants in four replicate conjugations plated in duplicate plates; approximately 1/40th of the total conjugation volume was plated, except for B. longum DJO10A-W3 where 1/400th was plated. §Average number of transconjugants per recipient from four replicate conjugations plated in duplicate; different letters indicate statistically significant differences between means ($P<0.01$).

**DISCUSSION**

Transformation through electroporation is a widely used technique due to its success in a large number of bacteria. To date, electroporation has been the only means of introducing plasmids into *Bifidobacterium* species. However, the published protocols are strain-dependent, requiring optimization for individual strains. Therefore, it is not surprising that these protocols were inefficient for the strains of interest in our laboratory. Conjugation could be a simple solution to this issue since conjugation protocols tend to be efficient for a wide range of hosts and are able to successfully transfer genetic material even between different phyla. The present work describes a robust conjugation protocol to be used in the transfer of genetic material from *E. coli* to several *Bifidobacterium* species. This represents the first confirmed report, to our knowledge, of a successful intergeneric conjugation into this genus. The system consists of a donor *E. coli* strain that can be easily excluded from the selection media by taking advantage of its DAP auxotrophy and a transmissible vector, pDOJHR-WD2, engineered with two Gram-positive antibiotic markers and the same 112 bp *oriT* sequence previously used in a *Streptomyces* species conjugation (Nikodinovic & Priestley, 2006).

Conjugation between *E. coli* and bifidobacteria was successful only when the mating occurred at 37 or 42 °C, aerobically on a buffered agar medium. Under these conditions, growth is impaired by the lack of DAP for *E. coli* and the presence of oxygen for bifidobacteria. Therefore, growth appeared to be unnecessary for the conjugation to occur, and it seems that anaerobic conditions, where bifidobacteria will be able to grow, inhibit the process. Since bifidobacteria are routinely grown under anaerobic conditions, this may be one of the reasons for unsuccessful conjugation attempts, as conjugation protocols call for mating conditions that favour the growth of the recipient strain.

When using the optimized protocol, the conjugation efficiencies observed initially ranged from $10^{-4}$ to $10^{-6}$ transconjugants per recipient, which are similar to reports in other *Actinobacteria*. Efficiencies of $10^{-4}$ to $10^{-8}$ have been reported for *Streptomyces* (Blaesing et al., 2005; Mazodier et al., 1989) and $10^{-6}$ for *Mycobacterium* (Gormley & Davies, 1991). Other groups have reported better efficiencies. These include for *Streptomyces* up to $10^{-2}$ (Luzhetskyy et al., 2006; Phornphisutthimais et al., 2010), for *Rhodococcus* $10^{-2}$–$10^{-7}$ (typically $10^{-3}$–$10^{-4}$) (Desomer et al., 1988) and for *Corynebacterium* up to $10^{-1}$ (Schäfer et al., 1994). From the initial conjugations into different bifidobacteria in this study, the highest conjugation efficiencies were obtained when the ratio of *E. coli* to bifidobacteria cells favoured *E. coli* (Table 3). When the ratio donor:recipient was further investigated for *B. animalis* subsp. *lactis* Bb-12 (Fig. 4), close to 100% of the recipients were conjugated when the ratio favoured *E. coli* 100 000 : 1, showing a dramatic improvement in the conjugation efficiency (>10 000 fold) when compared with a 1:1 ratio, which is typically suggested in conjugation protocols. Conversely, ratios that favoured the bifidobacteria strain showed low efficiencies and no transconjugants could be obtained once the ratio favoured bifidobacteria more than 100:1. This was not observed for plasmid transfer from *E. coli* to *Staphylococcus aureus* (Al-Masaudi et al., 1991) and therefore should be recognized as an important parameter for conjugal plasmid transfer between *E. coli* and bifidobacteria.

Plasmid incompatibility was defined more than 30 years ago (Novick et al., 1976) and experimental evidence supports it (reviewed by Nordström & Austin, 1989; Novick, 1987). However, we observed that in all cases the incoming vector (pDOJHR-WD2) coexisted with the resident plasmid (pDOJH10S) in *B. longum* DJO10A. Even in the case of strain DJO10A-W1, where the pDOJH10S plasmid was not visible by gel analysis, PCR and Southern blotting indicated that it was still present. The apparent reluctance to lose the pDOJH10S plasmid may suggest that it confers an advantage to the host. Small cryptic plasmids have previously been shown to confer fitness to a *Corynebacterium renale* host by an unexplained mechanism, but theorized to involve advantages accrued during coevolution of cryptic plasmids and the host cell (Srivastava et al., 2011). To investigate elements that could be advantageous for the strain, the pDOJH10S sequence was reanalysed. When analysing the sequence, it was evident that aside from the three ORFs previously annotated (*mob, rep* and *orfIV*) a new ORF was located overlapping by one nucleotide at the 3′ end of the *rep* gene. This newly identified ORF consisted of 324 base pairs and a BLASTP analysis with a translated sequence revealed homology with a gene predicted to be *repB* that has been identified in pBS423 from *B. longum* (Hirayama et al., 2012), pFI2576 from *B. longum* (Moon et al., 2009), pMB1 from *B. longum* (Rossi et al., 1996), pBC1 from *Bifidobacterium catenulatum* (Álvarez-Martin et al., 2007), pNV18 from *Nocardia*
(Chiba et al., 2007), pAL5000 from Mycobacterium fortuitum (Rauzier et al., 1988) and pRG01 from Propionibacterium acidipropionici (Kiatpapan et al., 2000). In all cases a similar gene organization is shared where this gene is immediately downstream and overlapping a repA gene, as now depicted for pDOJHR-W2 in Fig. 1. However, no evidence of potentially advantageous elements was found. It was also intriguing that DJO10A-W2, which was initially believed to be cured of both pDOJH10S and pDOJHR-W2, due to its sensitivity to chloramphenicol and lack of visible plasmids via gel electrophoresis, retained pDOJHR-W2 with a mutated cat gene, presumably caused by the ethidium bromide curing, as this has been reported previously for other Actinobacteria (Cramer et al., 1986). However, as both plasmids were subsequently lost during further curing experiments, it can be concluded that while the strain has an affinity for this cryptic plasmid, similarly to cryptic plasmids in other bacteria (Srivastava et al., 2011), it contains no essential functions for the organism and can be successfully eliminated without any noticeable deleterious effects.

The vector in strain DJO10A-W2 did allow a small, but statistically significant, increase in transfer efficiency compared with the wild-type, presumably due to a lower copy number of the plasmid ori. When the ori was completely removed from strain DJO10A-W3 the conjugation efficiency increased by one log, similar to the efficiency predicted for B. animalis subsp. lactis Bb-12 at a similar donor:recipient ratio (Fig. 4). In contrast, both the wild-type and DJO10A-W1 showed efficiencies below what was expected in B. animalis subsp. lactis Bb-12. This shows that, although pDOJH10S and pDOJHR-W2 can coexist in the host, there is a degree of incompatibility as expected for two plasmids with the same origin of replication.

In conclusion, a robust and simple conjugation method to transfer genetic material from E. coli into potentially any bifidobacterium was developed. This method could enable the transfer of large constructs and assist in the development of genetic engineering tools for the Bifidobacterium species.

ACKNOWLEDGEMENTS

The E. coli strain WD3064 (pBB109) vector was kindly provided by Dr Brett Barney (University of Minnesota, USA). The vector pRDR215 was kindly provided by Dr Shinji Masuda (Tokyo Institute of Technology, Japan). Dairy Management Inc. (DMI) is acknowledged for providing research funding for this study.

REFERENCES


Edited by: D. A. Mills