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A Stable and Efficient Transformation System for *Butyrivibrio fibrisolvens* OB156

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Abstract. A 9.5-kb shuttle vector capable of replication and selection in both *Escherichia coli* and *Butyrivibrio fibrisolvens* was constructed. Plasmid pUC118 provided replication functions and ampicillin resistance selection in *E. coli*. In *B. fibrisolvens*, replication was controlled by the native plasmid pRJF1 from strain OB156, and selectability was provided by a 3.5-kb fragment of plasmid pAMβ1 containing the erythromycin resistance gene. Optimum conditions for transformation were 15 kV/cm, 2 h recovery, and plating in an agar overlay on medium containing 10 μg erythromycin/ml. Maximum efficiency was 1.1×10^5 transformants per μg plasmid DNA (average 3×10^4), and restriction mechanisms reduced efficiency by a factor of 2×10^2. Nonselective growth for 200 generations gave no measurable loss of plasmid.

During the past decade considerable attention has been focused on the benefits to animal production that might result from genetic manipulation of rumen bacteria. Gains in productivity that could be obtained through potential improvements in the nutrition and health of ruminant animals have been widely discussed [4, 5, 15, 16, 19], but progress towards this goal has been hampered by a lack of the basic tools for genetic manipulation, such as stable vectors and efficient DNA delivery systems.

The two rumen bacterial species most frequently considered as recipients of new genetic material are the hemicellulolytic organisms *Prevotella ruminicola* [12, 20] and *Butyrivibrio fibrisolvens* [21, 22]. Vectors developed for *P. ruminicola* are showing great promise [12, 20], but progress has been somewhat slower with *B. fibrisolvens*.

There have been reports of limited success in the transfer of DNA into *B. fibrisolvens*. Teather [19] reported transfer of the broad-host-range plasmid RP4 from *E. coli* to *B. fibrisolvens* by conjugation, imparting ampicillin resistance to the recipient strain, and Hazlewood and Teather [7] described the transfer of broad-host-range plasmid pRK248 by PEG treatment of spheroplasts. However, because of plasmid instability these systems were not pursued. A more recent report of transformation of *B. fibrisolvens* by electroporation with a recombinant plasmid [21] demonstrated plasmid maintenance over a number of generations; however, the plasmids lacked a suitable selectable marker gene, which is essential for them to be employed as practical DNA transfer vectors. Whitehead and Hespell [9] have demonstrated transfer of the *Enterococcus faecalis* plasmid pAMβ1 to *B. fibrisolvens* by conjugation, but the large size of pAMβ1 (26.5 kb) makes it difficult to manipulate as a transformation vector. Subsequently, Whitehead [22] introduced pBS42, an *E. coli*/*Bacillus subtilis* shuttle vector, into *B. fibrisolvens* by electroporation. Although the feasibility of the system was demonstrated, transformation efficiency was low (25 transformants/μg DNA), and the use of this vector to transfer additional DNA has yet to be reported.

One reason for the difficulties encountered in developing transformation systems for *B. fibrisolvens* has been a shortage of genetic information available on this species. The cloning and sequencing of a small native plasmid from strain OB156 [8] provided the information needed to construct a shuttle vector without disturbing genetic elements of the plasmid that might be important for replication and stability. Use of a well-characterized plasmid that is native to *B. fibrisolvens* would also be expected to minimize the

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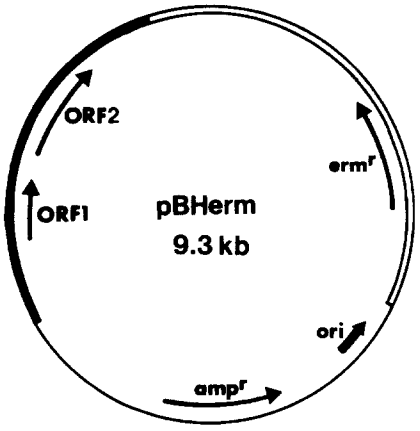

Fig. 1. Restriction map of the plasmid pBH*erm*. The narrow line represents pUC118 sequences; the thick, filled line, plasmid pRJF1; and the open line, DNA from plasmid pAMβ1.

problems of instability that are often associated with plasmids derived from other species.

This paper describes the development of a stable shuttle vector based on the *B. fibrisolvens* plasmid pRJF1, and its successful transfer to *B. fibrisolvens*.

Materials and Methods

Bacterial strains and growth conditions. DNA manipulations were performed in *E. coli* strain HB101 or DH5α, grown in LB medium or SOC medium [17] at 37°C. *B. fibrisolvens* strain OB156 was grown in either medium 10 [2] or in RF medium [11], under an atmosphere of CO_2:H_2 (96:4). Medium 10 was prepared with modifications as described by Teather [18] and further modified by replacing the carbon and energy sources with glucose, maltose, starch, and lactate (1 g/L of each; L-10 medium). Unless specified otherwise, ampicillin was used for selection in *E. coli* at a concentration of 100 μg/ml, and erythromycin at a concentration of 100 μg/ml in *E. coli* and 10 μg/ml in *B. fibrisolvens*. *B. fibrisolvens* OB156, OB157, and OB185 are Ottawa isolates. Strain Bu49 was isolated by Bryant and Small [1], and strains AR10, AR12, AR14, AR15, and AR27 were isolated from an Australian sheep by F. Hudman [10].

DNA isolation. Plasmid DNA preparations from *E. coli* were performed by alkaline lysis and purification through an anion exchange column according to the procedure described by the manufacturer (Qiagen Inc., California). Plasmid pBH*erm* was prepared from *B. fibrisolvens*, and pAMβ1 from *Enterococcus faecalis*, by alkaline lysis [17], except that incubation with 1 mg/ml lysozyme was included during the first step (20–40 min on ice for *B. fibrisolvens*; 10 min at room temperature for *E. faecalis*). Isolation of total DNA from strain OB156 was performed as described previously [10].

DNA manipulations. Restriction enzyme digests were performed under the conditions specified by the manufacturer (Boehringer Mannheim). DNA fragments were end-filled in Nick-translation buffer [14], with DNA polymerase I (Klenow fragment) and 40 μM concentrations of dATP, dGTP, dCTP, and dTTP at room temperature for 15 min. DNA was dephosphorylated where necessary with calf intestinal phosphatase under conditions specified by the manufacturer (Boehringer Mannheim). Phosphatase was inactivated by phenol/chloroform extraction.

Analytical gel electrophoresis used 1% (wt/vol) agarose gels in TBE buffer, and preparative gels used low-melting-point agarose in TAE buffer [17]. DNA was recovered from preparative gels by melting the agarose slice at 60°C, followed by phenol/chloroform extraction and ethanol precipitation.

DNA ligations used T4 DNA ligase in a buffer of 50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 5% polyethylene glycol, 1 mM ATP, 1 mM dTT, followed by incubation overnight at 16°C.

E. coli was transformed by electroporation as described by Dower et al. [3].

Transformation protocol for *B. fibrisolvens*. Twenty-five to 50-ml cultures of strain OB156 were grown overnight (approximately 16 h) from a 0.1% (vol/vol) inoculum, reaching an optical density of 1.0–1.3 (550 nm). Cells were chilled thoroughly on ice, centrifuged (3000 *g* for 10 min), and resuspended in 0.3 M sorbitol, 1 mM dTT, 1 μg/ml resazurin (electroporation buffer), under an atmosphere of 96% N_2:4% H_2. The cells were recentrifuged as before and resuspended in electroporation buffer to 0.2% of the original culture volume.

Forty-microliter aliquots of cells were mixed with plasmid DNA and pulsed with 1.0–2.5 kV from a 25 μF capacitor (bypass resistance 200 Ω), in a 1-mm path-length Bio-Rad electroporation cuvette. Cells were immediately transferred to RF medium (5 ml) and incubated at 39°C to allow recovery before plating, in an agar overlay, onto RF plates containing 10 μg erythromycin/ml.

Results

Shuttle vector construction. The cloning and characterization of the native plasmid from *B. fibrisolvens* strain OB156 (pRJF1) has been described previously [8]. The recombinant plasmid pBH3.3, which contains pRJF1 cloned into the *Hin*dIII site of pUC118, was used as the basis for shuttle vector construction.

pBH3.3 was digested with *Sma*I and ligated to an end-filled 3.5-kb *Hin*dIII fragment from pAMβ1, which encompasses an erythromycin resistance gene [13]. The new recombinant plasmid was designated pBH*erm* (Fig. 1).

Vector transfer into *B. fibrisolvens*. Transfer of pBH*erm* into *B. fibrisolvens* by electroporation demonstrated that the plasmid was able to replicate in *B. fibrisolvens* and conferred resistance to erythromycin at a very high level. Untransformed *B. fibrisolvens* grew in the presence of 10 ng/ml erythromycin, but were inhibited by 20 ng/ml. After transformation, growth occurred in concentrations up to 0.8 mg/ml and was inhibited by 1.0 mg/ml.

Following transformation of *B. fibrisolvens* OB156 with pBH*erm*, the resident native plasmid pRJF1 was no longer visible in DNA extracts from transformed bacteria (Fig. 2), having apparently been displaced from the cell. pBH*erm* was present at high copy number, as judged by the quantity of plasmid visible after gel electrophoresis of total DNA extracts, and

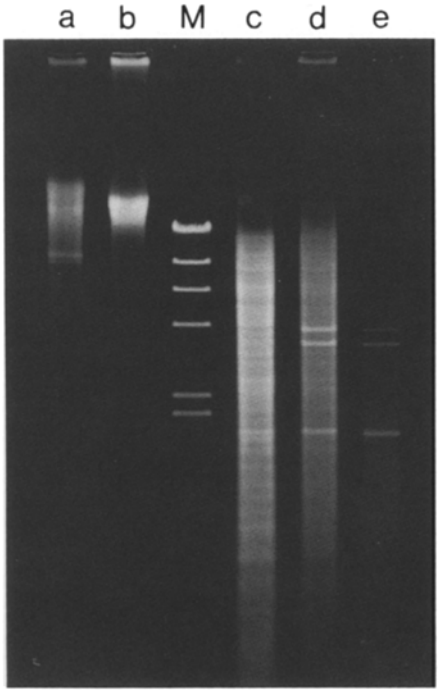

Fig. 2. (a) 0.25 μg of total DNA prepared from *B. fibrisolvens* OB156 after transformation with pBH*erm* compared with (b) 0.5 μg of total DNA from untransformed cells. The faint lower band in track (b) is native plasmid pRJF1, which appeared to be displaced after transformation with pBH*erm*. (c) *Eco*RI restriction digest of 2 μg of total DNA from untransformed OB156, compared with (d) 1.1 μg of total DNA from transformed OB156. DNA from the untransformed cells was loaded at approximately twice the concentration of that from transformed cells, to ensure that the absence of bands corresponding to pBH*erm* DNA was unequivocal. (e) pBH*erm* plasmid purified from *E. coli* and digested with *Eco*RI. Track M shows DNA size markers of bacteriophage λ DNA digested with *Hin*dIII.

yielded the predicted *Eco*RI digestion pattern (Fig. 2). It was concluded that no major plasmid rearrangements had occurred in the rumen bacterium.

Average transformation efficiency, with pBH*erm* isolated from *B. fibrisolvens* OB156 and recipient cells cultured in RF medium, was around 3×10^4 transformants per μg of plasmid DNA. However, the efficiency varied within a magnitude of 100-fold over eight experiments (highest efficiency $1.1 \times 10^5/\mu g$). When recipient cells were grown in L-10 medium, average efficiency was lower ($4–6 \times 10^3/\mu g$), but the variability between experiments was less (10-fold range in values over 11 experiments). When pBH*erm* was prepared from *E. coli*, average transformation efficiency of OB156 was reduced by about 2×10^2-fold, to give an average of $1.5 \times 10^2/\mu g$ for RF medium and $2 \times 10^1/\mu g$ for L-10 medium.

OB156 containing pBH*erm* was grown for 200 generations in the absence of erythromycin without detectable loss of the plasmid, demonstrating that the plasmid is highly stable in this host.

Table 1. Effect of recovery time on transformation efficiency of OB156 with plasmid pBH*erm*

Recovery time (h)	Transformants/μg
1	4.3×10^3
2	2.1×10^4
3	3.4×10^4
4	2.9×10^4

Optimization of transformation procedure. Initial transformation experiments used an electroporation buffer of 10% (vol/vol) glycerol, 1 mM dithiothreitol (dTT), and 1 μg/ml resazurin. Replacing the glycerol with 0.3 M sorbitol increased the transformation efficiency by approximately 12-fold.

The effects of post-electroporation recovery time are shown in Table 1, and 2–2.5 h was adopted as routine recovery time.

Figure 3A shows the effect of pulse strength upon transformation efficiency. Efficiency increased with increasing field strength up to 20 kV/cm with a Bio-Rad Gene Pulser. A setting of 15 kV/cm with a bypass resistance of 200 Ω was chosen for routine transformation because it minimized the likelihood of electrical arcing. When a BTX Electro Cell Manipulator 600 was used, voltage was limited to 12 kV/cm with a bypass resistance of 186 Ω, and efficiencies were marginally lower.

There was no significant difference in transformation efficiency among cultures harvested at various points in the growth curve between mid-logarithmic phase and stationary phase (Fig. 3B).

Cells that were prepared for electroporation and then snap-frozen in a methanol/dry-ice bath and stored at −70°C transformed at approximately the same efficiency as freshly prepared cells if used within 1–4 days, but dropped to around 10–30% efficiency at 30 days.

Butyrivibrio fibrisolvens Bu49, a strain which has different native plasmids and a different restriction modification system from OB156 (unpublished results), was also stably transformed by pBH*erm*. Strain AR14 was transformed at an efficiency similar to OB156. *B. fibrisolvens* strains ATCC19171, OB157, OB189, AR10, AR12, AR15, and AR27 were not transformed by pBH*erm* under conditions that would produce $> 10^4$ transformants with OB156.

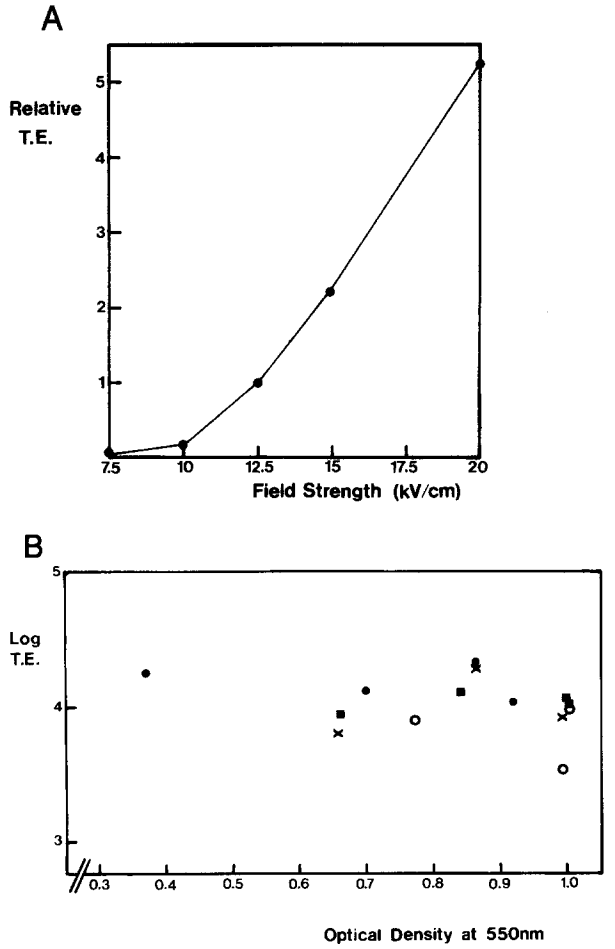

Fig. 3. Effects on transformation efficiency (T.E.) of OB156 (A) with varying pulse voltage, expressed as a proportion of the efficiency obtained at 12.5 kV/cm, i.e., 1.1×10^4 transformants/μg DNA. (B) Transformation efficiency of cells harvested for transformation at different stages of population growth. Each symbol represents data from a single experiment.

Discussion

The system described here provides a very promising method for genetically transforming *B. fibrisolvens* with a plasmid of high stability. The acceptable transformation efficiency and relatively low restriction barrier recommend strain OB156 and plasmid pBH*erm* as a tool for the study of the molecular genetics of this species. Our attempts to transform other *B. fibrisolvens* strains with pBH*erm* indicate that this plasmid may be suitable for around 30% of strains. Future work will include tests on additional *B. fibrisolvens* strains to establish more extensively the host range for pBH*erm*.

For testing the effect of growth phase on transformation efficiency, it was necessary to grow the cells in L-10 rather than RF medium. In RF medium the cells frequently displayed a tendency to aggregate during the logarithmic phase of growth and to disperse as they approached the stationary phase. Cultures harvested during the logarithmic phase were difficult to handle, apparently because of a sticky extracellular polysaccharide secretion that was later degraded. When grown in L-10 medium, aggregation did not occur, and it was possible to prepare electrocompetent cells at any stage of growth.

The features of the pBH*erm*/OB156 system meet the essential criteria for a routine transformation system and create the opportunity for more advanced genetic studies of *B. fibrisolvens*. Most importantly, the pBH*erm*/OB156 system has been shown to be a practical tool for making genetic alterations to the host organism [6].

ACKNOWLEDGMENT

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