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The large episomes of *Butyrivibrio proteoclasticus* B316^T have arisen through intragenomic gene shuttling from the chromosome to smaller *Butyrivibrio*-specific plasmids

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ABSTRACT

The genome of *Butyrivibrio proteoclasticus* B316^T contains three large episomes including a 302 kb chromid (BPc2) and two large plasmids of 361 (pCY360) and 186 kb (pCY186). The two plasmids are largely cryptic and it is therefore difficult to gauge their contributions or importance to the biology of *B. proteoclasticus*. Here, we provide evidence that at least BPc2 and pCY360 are essential as neither could be cured using several previously described curing techniques. We show that BPc2 exists at a copy number of 1, while pCY360 and pCY186 exist at copy numbers of 4 and 0.9, respectively. Yet the transcriptional activities of each episome are much less than that of the 3.5 Mb chromosome.

Codon usage analyses did not support the hypothesis that the genes of all three episomes were acquired horizontally. Instead our analyses suggest that the vast majority of genes on each episome were transferred from the 3.5 Mb *B. proteoclasticus* chromosome. Analysis of their replication origins, however, suggests the plasmid backbones share an evolutionary lineage with the smaller *Butyrivibrio* specific plasmids, pRJF1 and pRJF2. A survey of 13 species of the *Butyrivibrio*/*Pseudobutyrivibrio* assemblage identified similar large episomes in nine strains. DNA hybridization experiments revealed none contained an rRNA operon and only a 145 kb episome from *Pseudobutyrivibrio ruminis* possessed an ortholog of the pCY360 plasmid replication initiation protein. The size and distribution of episomes within the nine strains of *Butyrivibrio*/*Pseudobutyrivibrio* showed no correlation with 16S rRNA based phylogeny, leading to a hypothesis that the large episomes of *Butyrivibrio* spp., have arisen through intragenomic gene transfer events from the chromosome to small horizontally acquired elements.

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1. Introduction

Butyrivibrio proteoclasticus (formerly *Clostridium proteoclasticum*; Attwood et al., 1996; Moon et al., 2008) is a Gram-positive anaerobic butyrate-forming bacterium isolated from the rumen. It was originally isolated and studied because of its strong serine-type protease activity (Attwood et al., 1996), but has been investigated in more detail recently because of its contributions to hemicellulose degradation and the biohydrogenation of fatty acids (Wallace et al., 2006). Quantitative molecular techniques have shown *B. proteoclasticus* B316^T to be present at significant levels in rumen contents from animals consuming a variety of diets (Reilly and Attwood, 1998; Paillard et al., 2007). Recently the *B. proteoclasticus* genome was sequenced and found to be spread over four replicons

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including the 3.5 Mb chromosome and three large episomes of 361, 302 and 186 kb (Kelly et al., 2010). This is consistent with a previous observation of large episomal DNAs (approximately 2.5×10^8 Da or 410 kb in size) in a number of *Butyrivibrio fibrisolvens* strains (Teather, 1982). The *B. proteoclasticus* 302 kb replicon, designated BPc2 was determined to be a chromid based on the presence of two tRNAs (aspartic acid and threonine) and two complete ribosomal RNA operons (16S, 5S, and 23S rRNAs) (Kelly et al., 2010). The term chromid was applied to show BPc2 does not conform succinctly to the ideals of either a chromosome or plasmid (Harrison et al., 2010). In contrast, the 361 and 186 kb replicons designated pCY360 and pCY186 are largely cryptic with >75% of their ascribed open reading frames (ORFs) having no bioinformatically assignable function (Kelly et al., 2010). The % G + C content of each episome is almost identical to that of the major chromosome (Kelly et al., 2010) suggesting a long evolutionary relationship such that compositional bias of nucleotides have had sufficient time to adapt to those of the chromosome. While the *B. proteoclasticus* genome has been examined with a particular emphasis on hemicellulose degradation (Kelly et al., 2010), the episomal DNAs have not been subjected to detailed experimentation or bioinformatic analyses. We therefore sought to obtain greater insight into the potential contributions to, and the phylogenetic relationship of the episomes to the biology of *B. proteoclasticus*. Using copy number and plasmid curing experiments we find implicative evidence of the importance of the two largest episomes to the growth and/or survival of *B. proteoclasticus*. We show the episomes are transcriptionally active and confirm the majority of ORF designations through microarray analysis and investigate the distribution of other large episomal DNAs among species of *Butyrivibrio* and from the closely related genus, *Pseudobutyrivibrio*. Detailed phylogenetic and bioinformatic analyses are also reported and provide greater insight into their evolution and potential contributions to the biology of *B. proteoclasticus*.

2. Methods

2.1. Bacterial strains and media

All bacterial strains used in this study are listed in Table 1, and were grown under anaerobic conditions at 39 °C using M704 medium (DSMZ). For construction of recombinant plasmids *Escherichia coli* laboratory strain DH5α was used. *E. coli* was propagated on Luria–Bertani medium supplemented with 50 μg/ml ampicillin.

2.2. Bioinformatic analyses

The genome of *B. proteoclasticus* B316^T was sequenced and annotated by the Rumen Microbial Genomics team at AgResearch as described elsewhere (Kelly et al., 2010). The nucleotide sequence of the *B. proteoclasticus* B316^T genome is available in Genbank under Accession No. CP001810 (3.5 Mb chromosome), CP001811 (BPc2), CP001812 (pCY360) and CP001813 (pCY186). Sequences were orientated so nucleotides were numbered from the ribosome binding site of the *repB* gene. DNA secondary structures including inverse-, inverted- and direct-repeats were detected using an alignment of each replicon sequence against itself using an in-house standalone BLASTN program (Altschul et al., 1997). Codon usage analysis was performed using previously described modal codon usage software (Davis and Olsen, 2011). Major codons were determined using a custom Perl script. Comparisons were made to *Butyrivibrio crossotus* DSM2876 using the entire 24,82,791 bp of available draft sequence downloaded from Genbank (Accession No. ABWN00000000). Gene calls for the draft *B. crossotus* sequence were made using GLIMMER. Metabolic pathways were mapped and analyzed using iPath (Letunic et al., 2008; http://pathways.embl.de/). Microarray data was derived from previously reported microarray analyses (Leahy et al., 2010). Expression ratios were determined by comparing the median intensity of all genes among replicons following normalization. Normalization was performed in Limma (BioConductor; http://www.bioconductor.org) using the 'print-tip loess' procedure and is described along with all other quality control measures in (Leahy et al., 2010). Near identical results were seen between samples and when using the average as opposed to the median. Microarray results are available from the Gene Expression Omnibus (GEO) under GEO Accession No. GSE18716.

Table 1
Bacterial strains.

Genus	Species	Strain
Butyrivibrio	*crossotus*	DSM2876 (2)
	fibrisolvens	D1^T (2)
		C211 (1)
	hungatei	JK615 (3)
		A38 (2)
		C219 (1)
	proteoclasticus	B316^T (1)
		UC142 (3)
	Unspeciated	JK619 (3)
Pseudobutyrivibrio	*ruminis*	DSM9787 (5)
		CF3(4)
		CF1b(4)
	xylanivorans	Mz5^T(3)
		Ce52 (1)

Strains were obtained from the culture collections of: (1) AgResearch Ltd., Grasslands Research Centre, Palmerston North, New Zealand. (2) Dept. of Animal Sciences, University of Illinois at Urbana-Champaign, IL, USA. (3) Institute of Animal Physiology and Genetics, Prague, Czech Republic. (4) Ohio Agricultural Research and Development Center, Ohio State University, Wooster, OH, USA. (5) Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures), Germany.

2.3. Plasmid curing

Attempts to cure *B. proteoclasticus* of BPc2, pCY360 and pCY186 were based on previously described techniques that were successfully used in the curing of large plasmids. These included growth at higher than optimal temperatures (Morrison et al., 1983) or in M704 media supplemented with novobiocin (Kojic et al., 2005), acriflavine (Mesas et al., 2004), ethidium bromide (Mattarelli et al., 1994) or acridine orange (Sadowsky and Bohlool, 1983).

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