



Short communication: The complete genome sequence of *Bifidobacterium animalis* subspecies *animalis* ATCC 25527^T and comparative analysis of growth in milk with *B. animalis* subspecies *lactis* DSM 10140^T

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ABSTRACT

The objective of this work was to sequence the genome of *Bifidobacterium animalis* ssp. *animalis* ATCC 25527^T, the subspecies most closely related to *B. animalis* ssp. *lactis*, some strains of which are widely added to dairy foods as probiotics. The complete 1,932,963-bp genome was determined by a combination of 454-shotgun sequencing and PCR gap closing, and the completed assembly was verified by comparison with a *KpnI* optical map. Comparative analysis of the *B. animalis* ssp. *animalis* ATCC 25527^T and *B. animalis* ssp. *lactis* DSM 10140^T genomes revealed high degrees of synteny and sequence homology. Comparative genomic analysis revealed 156 and 182 genes that were unique to and absent in the *B. animalis* ssp. *animalis* genome, respectively. Among these was a set of unique clustered regularly interspaced short palindromic repeats (CRISPR)-associated genes and a novel CRISPR locus containing 30 spacers in the genome of *B. animalis* ssp. *animalis*. Although previous researchers have suggested that one of the defining phenotypic differences between *B. animalis* ssp. *animalis* and *B. animalis* ssp. *lactis* is the ability of the latter to grow in milk and milk-based media, the differential gene content did not provide insights to explain these differences. Furthermore, growth and acid production in milk and milk-based media did not differ significantly between *B. animalis* ssp. *lactis* (DSM 10140^T and B104) and *B. animalis* ssp. *animalis* (ATCC 25527^T). Growth of these strains in supplemented milk suggested that growth was limited by a lack of available low-molecular-weight nitrogen in the 3 strains examined.

Key words: *Bifidobacterium animalis* ssp. *animalis*, genome, growth in milk, clustered regularly interspaced short palindromic repeats (CRISPR)

Short Communication

Members of the genus *Bifidobacterium* are considered natural inhabitants of the human and other mammalian gastrointestinal tract, and numerous fermented dairy products are supplemented with bifidobacteria as a probiotic adjunct (Turroni et al., 2009). One widely used bifidobacterial species is *Bifidobacterium animalis*, which contains the 2 subspecies *lactis* and *animalis*. Generally, many strains within the *lactis* subspecies are regarded as technologically suitable for use as probiotics because they have been reported to be more acid-, bile-, and oxygen-tolerant than other members of the genus (Mainville et al., 2005; Jayamanne and Adams, 2006). Furthermore, *B. animalis* ssp. *lactis* has been reported to grow in milk and milk-based media, whereas *B. animalis* ssp. *animalis* has been reported to lack this ability (Meile et al., 1997; Masco et al., 2004). Some authors have speculated that *B. animalis* ssp. *lactis* diverged from *B. animalis* ssp. *animalis* and adapted specifically for growth in milk and that its genome has been streamlined for this specific niche (Lee and O'Sullivan, 2010). The ability to grow in milk is also thought to differentiate the 2 subspecies (Meile et al., 1997).

Although multiple fully sequenced genomes are available for *B. animalis* ssp. *lactis* (Barrangou et al., 2009; Kim et al., 2009; Garrigues et al., 2010; Sun et al., 2010; National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov/>), the genome of the most closely related subspecies, *B. animalis* ssp. *animalis*, has not been sequenced. Thus, the objective of this work was to completely sequence the genome of *B. animalis* ssp. *animalis* ATCC 25527^T and to compare it with that of *B. animalis* ssp. *lactis*. Additionally, the ability of both subspecies to grow in milk and milk-based media was assessed.

Bacterial Strains and Culture Conditions

The type strain of *B. animalis* ssp. *lactis* DSM 10140^T was obtained from the Deutsche Sammlung von Mi-

Received April 29, 2011.

Accepted August 16, 2011.

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krorganismen und Zellkulturen GmbH (DSM; The German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and grown on de Man, Rogosa, and Sharpe medium (de Man et al., 1960) supplemented with 0.05% (wt/vol) cysteine hydrochloride (**MRSC**). The commercial strain *B. animalis* ssp. *lactis* BI04 was obtained from Danisco USA Inc. (Madison, WI), and *B. animalis* ssp. *animalis* ATCC 25527^T was obtained from the American Type Culture Collection (ATCC; Manassas, VA). *Bifidobacterium*-specific primers developed by Kaufmann et al. (1997) were used with the following modified PCR protocol: 20 cycles of 94°C for 1 min, 57°C for 0.5 min, and 72°C for 1.5 min. Cultures of *B. animalis* ssp. *lactis* and *B. animalis* ssp. *animalis* were verified to the subspecies level using PCR primers and conditions designed by Ventura and Zink (2002).

Sequencing and Assembly of the *B. animalis* ssp. *animalis* Genome

Genomic DNA was isolated from 10 mL of overnight culture of *B. animalis* ssp. *animalis* ATCC 25527^T grown in MRSC using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). Genomic DNA was submitted for 454 pyrosequencing on a GS-FLX sequencer at The Pennsylvania State University. Sequencing was followed by de novo assembly in Newbler (Roche, Branford, CT). Following assembly, contigs were aligned using pheromone trail-based genetic algorithm (Zhao et al., 2008) using the *B. animalis* ssp. *lactis* DSM 10140^T genome as a reference (Barrangou et al., 2009). To close gaps, primers were designed on the end of each contig and PCR was conducted with appropriate primers. The PCR products were sequenced at the Huck Institute Genomic Core Facility at The Pennsylvania State University using 3' BigDye-labeled dideoxynucleotide triphosphates (v 3.1 dye terminators; Applied Biosystems, Foster City, CA) and a ABI 3730XL DNA analyzer with ABI sequence analysis software (version 5.1.1). Sequences were aligned and added to contigs to generate a completed circular genome using SeqMan (DNASTAR, Madison, WI).

A *KpnI* optical map of the *B. animalis* ssp. *animalis* ATCC 25527^T genome was generated at OpGen (Gaithersburg, MD). The assembled genome was aligned with the optical map using MapSolver (OpGen) to confirm genome assembly and with an in silico *KpnI* optical map of *B. animalis* ssp. *lactis* DSM 10140^T. The genome sequence of *B. animalis* ssp. *animalis* ATCC 25527^T was submitted for automated annotation at NCBI and deposited in GenBank (accession number CP002567).

Comparative Genomic Analysis

The genomic sequences of *B. animalis* ssp. *animalis* ATCC 25527^T and *B. animalis* ssp. *lactis* DSM 10140^T were aligned using the Mauve alignment tool with default settings (Darling et al., 2004). Differential gene content was identified using RAST (rapid annotations using subsystems technology; Aziz et al., 2008) as having less than 60% amino acid sequence identity (Chaudhuri et al., 2010) between sequences in the 2 genomes. Clustered regularly interspaced short palindromic repeats (**CRISPR**) were identified in the *B. animalis* ssp. *animalis* ATCC 25527^T genome using Dotter and CRISPRFinder (Grissa et al., 2007). The total number of SNP existing between the ATCC 25527^T and DSM 10140^T genomes was determined following alignment using Mauve. When protein sequences were aligned, BLASTP was used (Sayers et al., 2011).

Growth in Milk

Growth of *B. animalis* ssp. *animalis* and *B. animalis* ssp. *lactis* was evaluated in 10% reconstituted skim milk (**RSM**, Becton Dickinson, Franklin Lakes, NJ), RSM supplemented with 0.5% peptone and 1% yeast extract, and RSM supplemented with 0.5% casamino acids. Prior to inoculation of milk, cultures were prepared from frozen stock by growth in MRSC containing 2% (wt/vol) lactose at 37°C in an anaerobic incubator (85% N₂, 10% CO₂, and 5% H₂) for 16 to 18 h. One milliliter of turbid culture was then transferred to autoclaved 10% RSM and allowed to incubate anaerobically for 24 h. After activation, 1 mL of the 24-h culture was transferred to each treatment, mixed, and then incubated anaerobically at 37°C. Samples were taken initially and then every 6 h for 24 h for determination of pH and viable counts. Counts were made by pour-plating on MRSC with lactose agar and incubating anaerobically for 48 to 72 h. Two replications were performed. Mean increase in populations and decrease in pH were compared using ANOVA in Minitab (version 15, Minitab Inc., State College, PA).

Genome Sequencing and Comparison

The complete genome of *B. animalis* ssp. *animalis* ATCC 25527^T was 1,932,693 bp long, 5,790 bp shorter than that of *B. animalis* ssp. *lactis* DSM 10140^T (1,938,483 bp; Table 1). The assembly was verified by comparison with a *KpnI* optical map (Figure 1A) and only minor differences were observed between the in vivo and in silico optical maps, confirming that the assembly

Table 1. General characteristics of *Bifidobacterium animalis* strains

Organism	GenBank accession no.	Length (bp)	Coding (%)	G+C (%)	Genes (no.)	rRNA operons (no.)	tRNA (no.)	Insertion sequence elements (no.)
<i>B. animalis</i> ssp. <i>animalis</i> ATCC 25527 ^T	CP002567	1,932,963	85.4	60.47	1,597	4	52	4
<i>B. animalis</i> ssp. <i>lactis</i> DSM 10140 ^T	CP001606	1,938,483	90.3	60.48	1,629	4	51	6
<i>B. animalis</i> ssp. <i>lactis</i> B104	CP001515	1,938,709	90.5	60.48	1,631	4	52	6
<i>B. animalis</i> ssp. <i>lactis</i> Bb-12	CP001853	1,942,198	89.9	60.48	1,642	4	52	6
<i>B. animalis</i> ssp. <i>lactis</i> V9	CP001892	1,944,050	86.0	60.50	1,636	4	52	7
<i>B. animalis</i> ssp. <i>lactis</i> HN019 ¹	ABOT000000000	1,915,892	86.0	60.00	1,632	1	52	4
<i>B. animalis</i> ssp. <i>lactis</i> AD011 ²	CP001213	1,933,695	84.6	60.49	1,528	2	52	7

¹Currently exists as 28 contigs.

²Assembly and annotation issues might explain differences in size and number of genes.

of the *B. animalis* ssp. *animalis* ATCC 25527^T genome was accurate. Automated annotation from NCBI predicted that the genome of *B. animalis* ssp. *animalis* ATCC 25527^T contained 1,597 genes, 34 fewer than the 1,629 genes annotated in the genome of *B. animalis* ssp. *lactis* DSM 10140^T. The general characteristics of these 2 genomes along with the other sequenced *B. animalis* ssp. *lactis* strains appear in Table 1. Analysis of the 2 genomes for differential content revealed 156 and 182 genes in the *animalis* subspecies that were unique to and absent, respectively, compared with the *lactis* subspecies. Unique genes were identified as having less than 60% amino acid identity as detected by RAST (Aziz et al., 2008). The Mauve alignment showed a high degree of homology and synteny between the 2 genomes (Figure 1B) but also identified 73,021 SNP between the 2 genomes, indicating sequence diversity.

Differential content analysis did not reveal obvious genes or loci that may play a role in the ability of either organism to grow in milk. Genes potentially important for growth in milk were examined by protein-protein alignment in BLAST. Genes predicted to play a role in lactose transport and utilization [Balat_0475 (*lacS*)

and Balat_0476 (*lacZ*)] were evaluated and found to share 99% amino acid identity with their respective homologs (Banan_2470 and Banan_2475). Balat_1174, annotated as *pepO* and previously evaluated by Janer et al. (2005), was aligned with Banan_5790, and exhibited 98% amino acid sequence identity. Likewise, all other genes with predicted peptidase activity examined showed greater than 96% amino acid sequence identity, again suggesting a minimal difference in predicted function.

CRISPR

A novel CRISPR locus, Bana1, was identified in the genome of *B. animalis* ssp. *animalis* ATCC 25527^T, at 1,656 to 1,668 Mb, which contains 32 repeats and 30 spacers and 8 CRISPR-associated (*cas*) genes. The novel 29-bp CRISPR repeat 5'-GTTTGCCCCGCA-CAGGCGGGGATGATCCG-3' is homologous to repeats from the Ldbu1 CRISPR family (Horvath et al., 2009), and belongs to the Type IE CRISPR/Cas system (previously known as *E. coli* type), with universal *cas1* and *cas2* genes, as well as the signature Type I gene

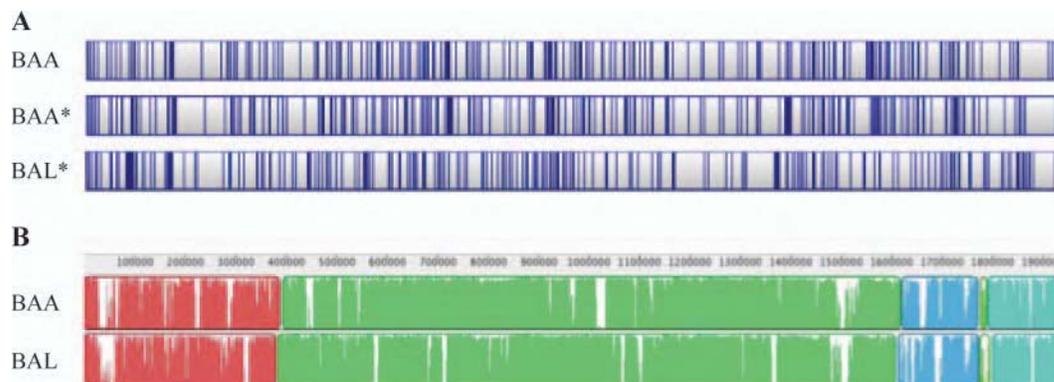


Figure 1. Comparisons of the complete genomes of *Bifidobacterium animalis* ssp. *animalis* ATCC 25527^T (BAA) and *B. animalis* ssp. *lactis* DSM 10140^T (BAL). (A) Experimental and in silico (*) *KpnI* optical maps of BAA and BAL. (B) Alignment of the complete genomes of BAA and BAL constructed using the Mauve alignment tool (Darling et al., 2004). Both figures begin at the same position on the BAA genome.

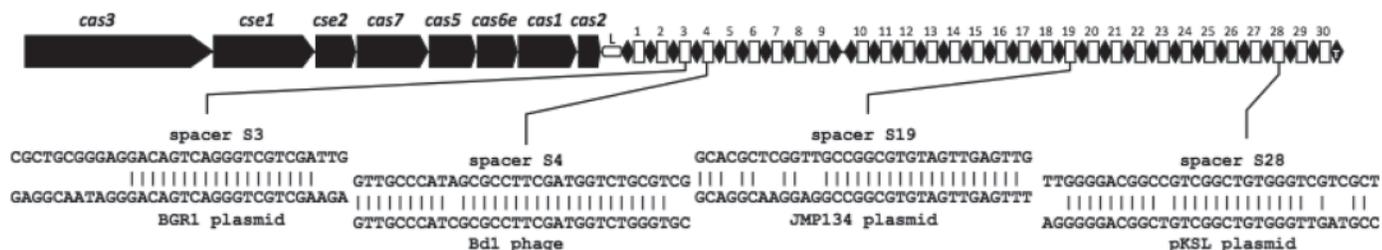


Figure 2. The *Bifidobacterium animalis* ssp. *animalis* ATCC 25527^T clustered regularly interspaced short palindromic repeats (CRISPR)/Cas system locus (*Bana1*) as it appears in the genome. The CRISPR-associated (*cas*) genes are located on the left followed by the leader sequence (designated L) and the repeat-spacer region. Repeats and spacers are designated as black diamonds and white boxes, respectively; the terminal repeat is indicated by a “T.” The sequences of spacers S3, S4, S19, and S28 were found to have significant homology with the indicated element.

cas3 (COG1203; Makarova et al., 2011). Although this CRISPR repeat sequence is novel, homologous 29-bp CRISPR repeats have been identified in the genomes of *Bifidobacterium gallicum* DSM20093, *Bifidobacterium catenulatum* DSM16992, and *Bifidobacterium angulatum* DSM20098. The repeat-spacer array is interrupted by an independently confirmed 77-bp random sequence located between the 10th and 11th repeats. Notwithstanding the paucity of sequence information available for bifidobacterial phages and plasmids, spacer S3 showed high homology to a tape measure protein from a *Bifidobacterium dentium* prophage sequence, and spacers S4, S19, and S28 showed homology to plasmid sequences from *Burkholderia*, *Ralstonia*, and *Streptomyces*, respectively (Figure 2). Additionally, several spacers showed homology to sequences from environmental genomic surveys, likely containing high levels of viral particles.

Although genome content, architecture, and synteny were high overall between *B. animalis* ssp. *animalis* and *B. animalis* ssp. *lactis*, several polymorphic islands provide insight concerning speciation. Interestingly, CRISPR content was hypervariable between the 2 subspecies, with the presence of a unique locus in each subspecies. Indeed, the *Bana1* and *Bala1* (Barrangou et al., 2009) loci are unique to *B. animalis* ssp. *animalis* and *B. animalis* ssp. *lactis*, respectively. This is consistent with CRISPR locus hypervariability previously observed in bifidobacteria (Briczinski et al., 2009; Horvath et al., 2009) and their susceptibility to horizontal gene transfer. Together with spacer sequence homology to foreign genetic elements, notably a *Bifidobacterium* prophage sequence (Ventura et al., 2009), this is consistent with the involvement of CRISPR/Cas systems in providing defense against invasive genetic elements (Barrangou et al., 2007; Horvath and Barrangou, 2010). The occurrence of different CRISPR/Cas systems in closely related organisms is likely an indicator of the selective pressure that foreign genetic elements, such as phages, apply on these bacterial hosts (Andersson and Banfield,

2008; Tyson and Banfield, 2008; Horvath and Barrangou, 2010). The critical role that CRISPR/Cas systems may play in bifidobacteria is also highlighted by the notable absence of other phage-resistance mechanisms such as restriction modification and abortive infection (Ventura et al., 2009).

Comparison of Growth in Milk

Growth of *B. animalis* ssp. *lactis* and *B. animalis* ssp. *animalis* ATCC 25527^T in milk and supplemented milk was evaluated over 24 h (Figure 3). The increase in population was not statistically different when compared within each of the milk-based media evaluated. Populations of *B. animalis* ssp. *animalis* and *B. animalis* ssp. *lactis* increased by approximately 1 log after 24 h incubation in unsupplemented RSM. Populations in the supplemented milks (for all 3 organisms) were similar and increased by approximately 2 to 2.5 logs (10-fold greater), a statistically significant increase when compared with growth in unsupplemented RSM ($P < 0.001$).

Acid production (pH) was also monitored over 24 h to assess the ability of *B. animalis* ssp. *animalis* and *B. animalis* ssp. *lactis* to acidify milk-based media. Organisms grown in milk reduced the pH by approximately 0.5 pH units after 24 h (no statistical difference between organisms; final pH ~5.9). Organisms grown in milk supplemented with 0.5% casamino acids or 0.5% peptone and 1% yeast extract decreased the pH by 2.0 to 2.5 units after 24 h (pH approximately 4.3) except for DSM 10140^T grown in 0.5% casamino acids, which only decreased the pH by approximately 1.5 units. Although DSM 10140^T grew to similar levels as ATCC 25527^T and B104 in milk supplemented 0.5% casamino acids, the growth resulted in a significantly smaller reduction of pH ($P < 0.001$).

Supplementation of RSM with casamino acids, a source of amino acids and peptides from casein, stimulated growth of both *B. animalis* ssp. *animalis* and *B.*

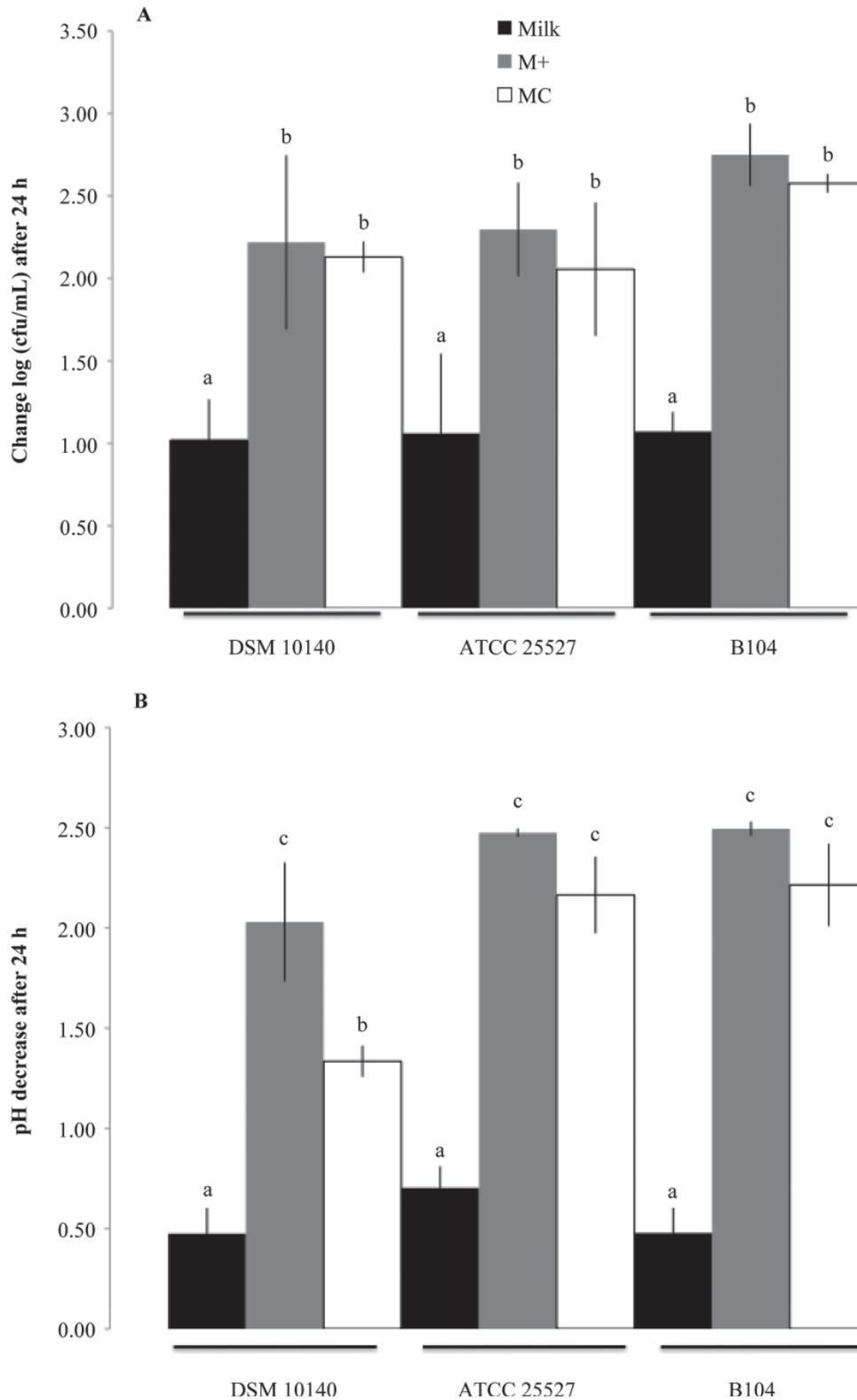


Figure 3. Evaluation of growth in milk and milk supplemented with 0.5% casamino acids or supplement with 0.5% peptone and 1% yeast extract. (A) Change in population (log cfu/mL) of each organism after anaerobic incubation for 24 h at 37°C in the indicated medium; (B) pH decrease after 24 h. Letters represent statistically significant differences after 24 h ($P < 0.05$). Milk = 10% reconstituted skim milk (RSM); M+ = 10% RSM + 1% peptone + 0.5% peptone; MC = 10% RSM + 0.5% casamino acids. Initial levels for each inoculation were approximately 3.0×10^6 cfu/mL.

animalis ssp. *lactis*, suggesting that proteolytic activity is limiting for growth in milk. This finding differs from the data reported previously by Masco et al. (2004), who observed an increase of 2.0 to 2.5 log cfu/mL after 24 h for *B. animalis* ssp. *lactis* strains grown in RSM.

The study provides insight into the genetic diversity that exists in the *B. animalis* species. Differential gene content did not provide a clear explanation for phenotypic differences alleged between the 2 subspecies. A novel CRISPR locus, Bana1, containing 32 repeats and 30 spacers, was identified in the genome of *B. animalis* ssp. *animalis* ATCC 25527^T. In addition, and in contrast to previous reports, growth of *B. animalis* ssp. *animalis* ATCC 25527^T in milk did not statistically differ from growth of *B. animalis* ssp. *lactis* DSM 10140^T or BI04. It appears that *B. animalis* ssp. *animalis* and *B. animalis* ssp. *lactis* both lack the necessary proteolytic activity required for good growth in milk.

ACKNOWLEDGMENTS

J.R.L. was funded, in part, by the Penn State University Microbial Functional Genomics USDA-AFRI Training Grant (Contract 2010-65110-20488). The authors thank Stephan Schuster (Pennsylvania State University, University Park) for assistance with 454-sequencing and assembly, and the Penn State Genomics Core Facility (University Park) for Sanger sequencing.

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