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Identification of *Brevibacteriaceae* by Multilocus Sequence Typing and Comparative Genomic Hybridization Analyses▽†

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Multilocus sequence typing with nine selected genes is shown to be a promising new tool for accurate identifications of *Brevibacteriaceae* at the species level. A developed microarray also allows intraspecific diversity investigations of *Brevibacterium aurantiacum* showing that 13% to 15% of the genes of strain ATCC 9174 were absent or divergent in strain BL2 or ATCC 9175.

Brevibacteriaceae play a major part in the cheese smear community (6, 11). The classification and typing of cheese-related *Brevibacteriaceae* have been based mainly on molecular methods such as amplified ribosomal DNA restriction enzyme analysis, pulsed-field gel electrophoresis, and ribotyping (8, 10, 12). Recently, the original *Brevibacterium linens* group was split into two species on the basis of their physiological and biochemical characteristics, the sugar and polyol composition of their teichoic acids, and their 16S rRNA sequence and DNA-DNA hybridization levels. One species remains *B. linens* and is represented by type strain ATCC 9172. The other, represented by type strain ATCC 9175, has been renamed *Brevibacterium aurantiacum*. Regarding this new classification, the taxonomic position of cheese-related isolates has to be revisited and potential relationships between phylogenetic affiliation and the potential occurrence of given metabolic characteristics redefined (7). The unfinished genome sequence of *B. aurantiacum* ATCC 9174 has recently been released by the Joint Genome Institute (http://genome.jgi-psf.org/draft_microbes/breli/breli.home.html). The development of focused phylogenetic approaches using multiple markers in conjunction with whole-genome screening techniques such as comparative genomic hybridization (CGH) has proven to be useful for the detailed characterization of pathogenic species, including food pathogens (3, 5, 9). However, only a few technological species have been investigated at an intraspecies level (2). Our intention was thus to develop modern tools to facilitate the typing of strains of technological interest, for which *Brevibacteriaceae* could be used as a case study.

Phylogenetic analysis of cheese-related *Brevibacteriaceae* shows an organization in three main branches. Three cheese *Brevibacterium* sp. strains, BL2, CNRZ918, and ATCC 9174, and *B. aurantiacum* and *B. linens* type strains ATCC 9175 and ATCC 9172, respectively, (7), were analyzed both by 16S rRNA sequencing with the universal primers and by multilocus sequence typing (MLST). The 16S rRNA analysis showed the phylogenic relationship between these strains (Table 1). *B. linens* ATCC 9172 is an independent lineage. BL2 and ATCC 9174 were related to *B. aurantiacum* type strain ATCC 9175. Interestingly, strain CNRZ918 presents similarities to the *B. aurantiacum* lineage, but this strain appeared to be closely related to *Brevibacterium antiquum*, with 99% identity between their 16S rRNA sequences.

To extend the 16S rRNA phylogenetic organization of technological *Brevibacterieceae*, an MLST approach was used. Nine genes (*cysN*, *glnA*, *gyrA*, *metY*, *metX*, *mgl*, *pheS*, *sahH*, and *tkt*) were chosen from the conserved housekeeping genes involved in sulfur, carbon, or nitrogen metabolism or general cellular processes. For each gene, we designed primers (Table 1) within the conserved part surrounding a variable area after sequence alignment of these genes from *B. aurantiacum* ATCC 9174, *Arthrobacter aurescens* (TC1), and *Arthrobacter* sp. strain FB24. Among these genes, *sahH* was especially discriminating at both the interspecific and intraspecific levels, showing as much as 3% sequence divergence between *B. aurantiacum* ATCC 9174 and ATCC 9175 (Table 1). The discriminating ability of *glnA* and *cysN* was more questionable, while the *gyrA*, *metY*, *metX*, and *tkt* markers were discriminating only at an interspecific level (Table 1). After concatenation of the nine DNA sequences, the phylogenetic tree obtained shows three MLST clusters (Fig. 1). Cluster I contained *B. aurantiacum* ATCC 9175, ATCC 9174 and BL2. Cluster II corresponded to strain CNRZ918, and cluster III was composed of the *B. linens*

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† Supplemental material for this article may be found at http://aem.asm.org/.

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TABLE 1. 16S and MLST markers used in this study[a]

Locus	Putative gene product	Sequence[b] (5′–3′)	Amplicon size (bp)	% Sequence difference from ATCC 9174			
				ATCC 9175	BL2	ATCC 9172	CNRZ918
cysN (BL2148)	Sulfate adenylyltransferase	F: GCTTCGCCACGGCCGGTTCCG R: GGCGACGGGTCTGCTCGGTCGCA	366	0	0	2.3	0
glnA (BL3684)	Glutamate synthase	F: ACCTTCTCGCTGCTGCCTTG R: GAGTGAGACGTGTGTGTGCAT	483	0	0	18.4	0
gyrA (BL1402)	DNA gyrase, subunit A	F: GAGAACTTCTCCGCGAACATG R: CTCCGGAATGAGGTCTTCCAT	467	0	9.2	13.7	9.2
metX (BL36)	Homoserine *O*-acetyltransferase	F: ATGCCGTCATCGGCGGATCGATGG R: GCCTCGGTTTCGGTGAGGAAGCCGTCATG	610	0	0	19.7	11.6
metY (BL56)	*O*-Acetylserine sulfhydrylase	F: AACATCGCCGAAGCCGGTGACCAC R: ACCCTGCACACGGGCCTTGAG	540	0	0	18.3	18.5
mgl (BL613)	Methionine γ-lyase	F: AATGAATACGAGCGCTGG R: CTCGGGGCGGCTCTCCAACCA	507	3.1	1.3	0	12.4
pheS (BL2678)	Phenylalanyl tRNA synthase	F: CGCCTGAACTTCGATTCCCTCAAC R: CATGCCGAAGGCGAAGCCCTG	507	0	1.7	7.8	4.5
sahH(BL3170)	*S*-Adenosyl-L-homocysteine hydrolase	F: GTTGACGTTGATGGCCGGGAA R: TGCAACATCTTCTCCACCCAG	441	3	0	24.1	12.1
tkt (BL2672)	Transketolase	F: ACTCGCCCCTACAGCGGCACCTTC R: GTCCGCGGGCTGCGCGTCGAACCA	660	0	0	14.5	9.5
16S rRNA gene	rRNA	F: AGAGTTTGATCCTGGCTCAG R: AAGGAGGTGATCCAGCCGCA	1,541	0.3	0.2	3.8	2.6

[a] For each gene, its putative function, the set of corresponding PCR primers, the amplicon size, and the percentage of sequence divergence are indicated. Gene prediction and functional annotation were performed with AGMIAL (http://genome.jouy.inra.fr/~vloux/publications/forquin08/).
[b] F, forward; R, reverse.

ATCC 9172 type strain. Similarities of 92.3% and 86.5% were observed between the MLST sequence of ATCC 9174 and those of CNRZ918 and ATCC 9172, respectively. We can propose *tkt* as a marker for interspecies discrimination. According to 16S rRNA and MLST data, the original classification of cheese-related *Brevibacteriaceae* into two different species was also confirmed and extended. Indeed, the occurrence of *B. antiquum* had previously been restricted to permafrost, while this study constitutes the first report of a *B. antiquum*-related cheese isolate (7). Interestingly, cheese-related *Brevibacteriaceae* bacteria were of a polyphyletic origin, suggesting that adaptation to the cheese habitat could have occurred several times independently in various lineages of *Brevibacteriaceae*.

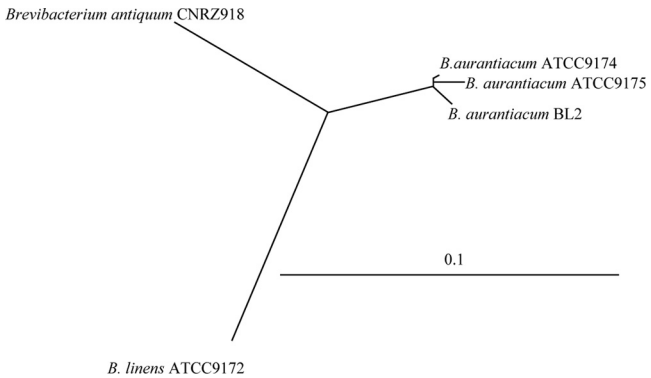

FIG. 1. Phylogenic tree based on DNA sequences of concatenated MLST markers. The sequences of the nine genes were concatenated in length. The phylogenic tree was constructed with concatenated nucleotide sequences of nine housekeeping genes (*cysN*, *glnA*, *gyrA*, *metY*, *metX*, *mgl*, *pheS*, *sahH*, and *tkt*) to obtain contigs of 4,581 bp and was displayed with the Treeview X software in an unrooted format.

Assessing *B. aurantiacum* genome variability on the basis of CGH analysis. In order to extend our analysis of cheese-related *Brevibacteriaceae*, we performed a genome comparison by hybridization analysis with the five strains studied by MLST. For this purpose, we used a microarray based on the unfinished genome sequence of *B. aurantiacum* ATCC 9174 (see the supplemental material). The array was further hybridized with the DNAs from the three cheese-related *Brevibacterium* groups as defined by MLST. The CGH analysis was performed by using the Franck Picard model (13). Although a signal was obtained with DNAs from ATCC 9172 and CNRZ918, the $\log_2$ ratio was not significantly discriminating (see Fig. S1 in the supplemental material). The use of arrays should therefore be restricted to investigation of the genomic variability of *B. aurantiacum* species. Eighty-five percent and 87% of the probes gave a positive signal with *B. aurantiacum* ATCC 9175 and BL2, respectively.

TABLE 2. Distribution among major functional groups of the variable part of the *B. aurantiacum* ATCC 9174 genome

Function	No. of regions (no. of genes) deleted or divergent in:	
	ATCC 9175	BL2
Involvement in:		
Biosynthesis	1 (22)	1 (21)
Unknown function	7 (70)	17 (106)
Catabolic pathways	46 (427)	23 (304)
Heavy metal resistance	8 (117)	8 (94)
Antibiotic resistance	0 (0)	1 (10)
Oxidative stress response	1 (14)	2 (22)
Total	62 (650)	52 (557)
Mobile genetic elements		
Transposon related	29 (328)	19 (225)
Phage related	2 (41)	2 (33)
Total	31 (369)	21 (258)

Among the 4,211 genes of ATCC 9174 for which probes were present, 3,308 were conserved in both ATCC 9175 and BL2. Five hundred fifty-seven and 650 genes were probably absent or highly divergent in BL2 and ATCC 9175 compared to ATCC 9174, respectively. Table 2 summarizes the functional groups associated with the divergent parts of the *B. aurantiacum* genome and the mobile genetic element-related genes associated. We further focused our investigations on the variable parts of the genome with potential links with the adaptation of *B. aurantiacum* to cheese characteristics such as salt resistance, peptide transport, and amino acid- or sulfur-related metabolism. The presence or absence of these regions (see Table S1 in the supplemental material) was further checked by PCR when possible. A first deleted region spanning the region from BL614 to BL621 in the genome of strain ATCC 9174 was confirmed by PCR amplification. By sequencing these PCR fragments, we determined that the ends of the fragments deleted in strains ATCC 9175 and BL2 were conserved (Fig. 2B). This observation suggests an insertion of a catabolic island in the genome of strain ATCC 9174. This chromosomal region encodes an oligopeptide-type ABC transporter (BL618 to BL621), two putative monooxygenases (BL615, BL617), and a reductase (BL616) that could be involved in sulfonate catabolism (Fig. 2A). The CGH results also showed that five peptide or amino acid transporters are deleted in both the ATCC 9175 and BL2 genomes while an additional peptide transporter (BL1002 to BL1006) seems to diverge between ATCC 9174 and BL2 (see Table S1 in the supplemental material). The diversity among the peptide uptake systems could be of potential interest for biotechnological applications. Indeed, yeast (*Geotrichum candidum*) and bacteria present during the cheese-ripening process degrade casein and produce peptides (1, 4). These peptides can be further used by other microorganisms for volatile sulfur compound production. The microarray developed in this study offers opportunities for further studies aiming at evaluating the biodiversity of a

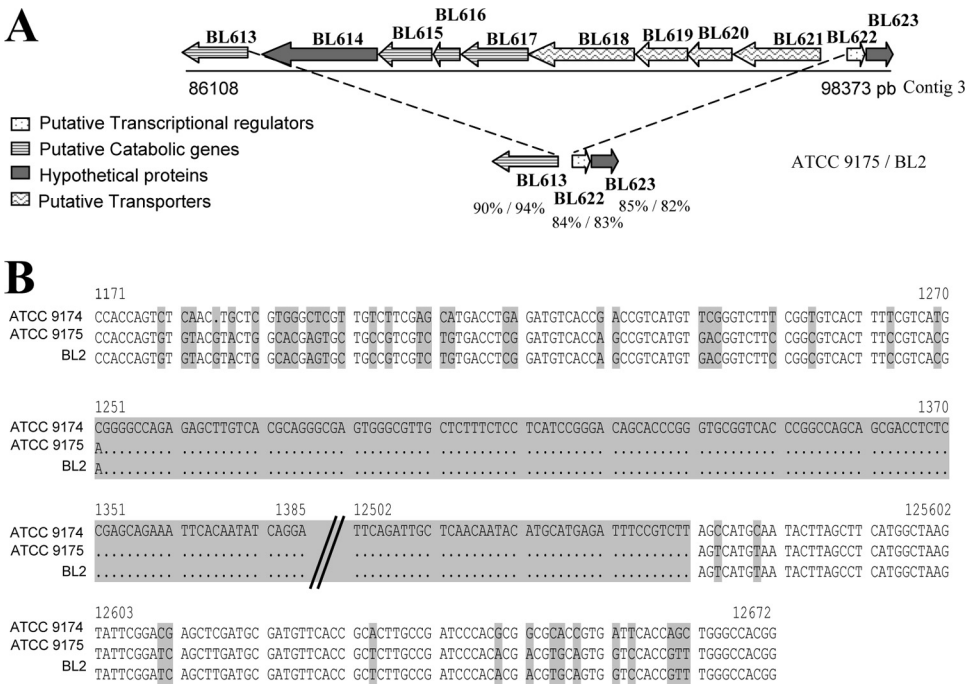

FIG. 2. Variable region spanning the region from BL614 to BL621 in the *B. aurantiacum* ATCC 9174 genome and lacking in the corresponding genomic regions of strains ATCC 9175 and BL2. (A) Schematic representation of the region from BL613 to BL623 in the genome of ATCC 9174 and of the same region of strains ATCC 9175 and BL2. The percent similarities of the BL613, BL622, and BL623 genes between strain ATCC 9174 and strains ATCC 9175 and BL2 are indicated. (B) Alignment of the DNA sequences adjacent to the variable chromosomal region between BL614 and BL621 in strains ATCC 9174, ATCC 9175, and BL2 and determination of the borders of the insertions or deletions. Variable nucleotides are shaded.

higher number of *B. aurantiacum* strains of technological interest, coming from various cheese habitats.

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