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Multilocus variable-number of tandem repeat analysis (MLVA) for *Clostridium tyrobutyricum* strains isolated from cheese production environment

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ABSTRACT

Clostridium tyrobutyricum is a gram-positive spore-forming anaerobe that is considered as the main causative agent for late blowing in cheese due to butyric acid fermentation. In this study, multilocus variable-number of tandem repeat (VNTR) analysis (MLVA) for *C. tyrobutyricum* was developed to identify the source of contamination by *C. tyrobutyricum* spores in the cheese production environment. For each contig constructed from the results of a whole genome draft sequence of *C. tyrobutyricum* JCM11008^T based on next-generation sequencing, VNTR loci that were effective for typing were searched using the Tandem Repeat Finder program. Five VNTR loci were amplified by polymerase chain reaction (PCR) to determine their number of repeats by sequencing, and MLVA was conducted. 25 strains of *C. tyrobutyricum* isolated from the environment, raw milk, and silage were classified into 18 MLVA types (DI = 0.963). Of the *C. tyrobutyricum* strains isolated from raw milk, natural cheese, and blown processed cheese, strains with identical MLVA type were detected, which suggested that these strains might have shifted from natural cheese to blown processed cheese. MLVA could be an effective tool for monitoring contamination of natural cheese with *C. tyrobutyricum* in the processed cheese production environment because of its high discriminability, thereby allowing the analyst to trace the source of contamination.

1. Introduction

Clostridium tyrobutyricum is a gram-positive spore-forming anaerobe that is considered as the main causative organism responsible for late blowing in cheese due to butyric acid fermentation (Bergeres and Sivela, 1990; Klijn et al., 1995; Le Bourhis et al., 2007). Late blowing is caused by the outgrowth of *C. tyrobutyricum* spores during the ripening period of cheese. The characteristics of late blowing include the production of acetic acid, butyric acid, a large quantity of gas associated with production of carbon dioxide, and hydrogen, as well as the development of a bad odor. Processed cheese is prepared from crushed hard or semi-hard cheese such as Cheddar and Gouda and by adding ingredients, including whey powder, milk powder, cream, butterfat, emulsifier, salt, seasonings and water, heating at 80 °C or higher and thorough mixing. Processed cheese has been largely produced in the United States, Russia and Japan. Especially in Russia and Japan, processed cheese dominates the large part of the total cheese consumption (Sorensen, 2005). Spoilage of processed cheese due to butyric acid fermentation also often occurs because *C. tyrobutyricum* spores can survive the conditions of cheese processing (Loessner et al., 1997). In most cases, such blown processed cheese loses its market value, leading to significant economic loss.

C. tyrobutyricum spores are detected in the environment, for example, in soil, water, air, and unhygienic animal bedding. The main source of contamination of raw milk is thought to be poor quality silage (Stadhouders and Spoelstra, 1990; Julien et al., 2008). *C. tyrobutyricum* spores, which can pass through alimentary tract of the cow, are excreted within the feces to the farm environment. Transmission to milk occurs *via* fecal contamination of the cow's teats. (Stadhouders and Spoelstra, 1990). Among *Clostridium beijerinckii*, *Clostridium sporogenes*, *Clostridium butyricum*, and *C. tyrobutyricum*, which are capable of butyric acid fermentation, *C. tyrobutyricum* is most commonly isolated from late-blown cheese (Cocolin et al., 2004; Le Bourhis et al., 2005). However, no specific culture medium that can distinguish each *Clostridium* species is available and phenotypic discrimination is almost impossible. Thus, it is difficult to identify each strain (Ingham et al., 1998; Sperner et al., 1999). Recently, more reliable techniques using PCR-based methods (Klijn et al., 1995; Herman et al., 1995; Cremonesi et al., 2012) and 16S rRNA probe-based hybridization methods (Klijn et al., 1994; Knabel et al., 1997) have been developed to identify *C. tyrobutyricum*. Such techniques are required to identify contaminants of natural cheese when late blowing of cheese occurs. However, it is often difficult to obtain reliable information on the

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actual source of contamination using the results of species-level identification.

Generally, the identification of the contaminating agent at the strain level requires DNA typing. Most of the previous reports on DNA typing of *Clostridium* are limited to pathogenic strains such as *Clostridium difficile*, *Clostridium botulinum*, and *Clostridium perfringens* (Alonso et al., 2005; Hielm et al., 1998; Chalmers et al., 2008). For *C. tyrobutyricum*, although late blowing of cheese leads to considerable economic losses, reports employing DNA typing for strain identification have been limited.

Pulsed-field gel electrophoresis (PFGE) is considered the gold standard of DNA typing. Although PFGE is a complicated technique and is time consuming, it is widely used for a number of bacterial strains because of its high discriminability and reproducibility. However, for *C. tyrobutyricum*, several problems of PFGE, such as smearing of PFGE bands, have been reported (Garde et al., 2012), and improved protocol with no difficulty has not yet been established. Previous reports have examined *C. tyrobutyricum* strains isolated from milk and cheese using PFGE (Ingham et al., 1998; Christiansen et al., 2005; Garde et al., 2012). However, those reports did not include any discussion of the source of contamination or comparison of the discriminatory power of various DNA typing techniques.

Multilocus variable-number of tandem repeat (VNTR) analysis (MLVA) is a DNA typing technique employed to detect the number of repeat units in repetitive loci that are interspersed along genomic DNA. Gel electrophoresis or sequence analysis of the PCR-amplified product of each VNTR locus is conducted to detect the number of repeats. MLVA is thus a DNA typing technique that is simple, rapid, and highly reproducible. Although earlier studies using MLVA have extensively examined pathogens such as *Staphylococcus aureus* (Sabat et al., 2003), *Listeria monocytogenes* (Murphy et al., 2007; Miya et al., 2008), and *C. botulinum* (Fillo et al., 2011), reports on food spoilage organisms other than *Geobacillus* species (Seale et al., 2012) and *Bacillus licheniformis* (Dhakal et al., 2013), which are isolated from milk powders, are scarce.

To our knowledge, this study is the first to use MLVA of *C. tyrobutyricum* to trace the source of contamination during cheese production with high discriminability and reproducibility. In addition, we also validated the effectiveness of the MLVA with respect to the management of natural cheese during cheese production.

2 . Materials and methods

2.1 . Bacterial strains

C. tyrobutyricum JCM11008^T was used to generate the whole genome draft sequence. Table 1 lists the 25 strains used for the development of MLVA. Of these strains, two strains were purchased from the National Collection of Industrial Food and Marine Bacteria (NCIMB, Scotland, UK). Table 2 shows the 29 strains used to trace the source of contamination.

2.2 . DNA extraction

C. tyrobutyricum strains were cultured in GAM semisolid without dextrose "Nissui" medium (Nissui Pharmaceutical, Tokyo, Japan) and filtered to remove the agarose, at 35 °C in anearobic condition. For JCM11008^T, DNA extraction was conducted using phenol-chloroform extraction (Takahashi et al., 2004). In the other strains, DNA extraction was conducted using NucleoSpin Tissue Kit (MACHEREY-NAGEL GmbH & Co. KG, Duren, Germany) according to the manufacture's protocols.

2.3 . Whole genome shot gun sequencing and de novo assembly

The whole genome shot gun sequencing was performed using the Roche GS junior platform (Roche, Basel, Switzerland), the GS junior Rapid Library preparation kit, and GS junior emPCR kit (Lib-L), following the manufactures' protocols. Construction of contigs was performed by using the GS *De novo* assembler (Roche, Basel, Switzerland). These sequences were annotated using Genome Traveler (In Silico Biology, Kanagawa, Japan).

Table 1
MLVA profile of the strains used for calculation of the DI value.

Isolate no.	Origin	Isolation date (day/mo/year)	TR1	TR2	TR3	TR4	TR5	MLVA type
IFH 2477	Environment	17-Dec-12	11	5	4	6	8	TYR 1
IFH 2479	Environment	25-Dec-12	11	5	4	6	8	TYR 1
IFH 2491	Environment	21-Jan-13	11	5	4	6	8	TYR 1
IFH 2476	Raw milk	25-Dec-12	11	5	4	6	8	TYR 1
IFH 2483	Environment	25-Dec-12	10	5	4	6	8	TYR 2
IFH 2493	Environment	21-Jan-13	10	5	4	6	8	TYR 2
IFH 2496	Environment	21-Jan-13	10	5	4	6	8	TYR 2
IFH 2487	Environment	17-Dec-12	2	7	6	6	7	TYR 3
IFH 2497	Environment	21-Jan-13	2	7	6	6	7	TYR 3
IFH 2484	Environment	17-Dec-12	9	8	4	6	8	TYR 4
IFH 2475	Raw milk	14-Dec-12	9	8	4	6	8	TYR 4
IFH 2492	Environment	21-Jan-13	7	7	4	7	7	TYR 5
IFH 2480	Environment	17-Dec-12	3	6	4	7	8	TYR 6
IFH 2481	Environment	17-Dec-12	4	6	6	6	7	TYR 7
IFH 2486	Environment	17-Dec-12	4	6	4	7	8	TYR 8
IFH 2488	Environment	17-Dec-12	5	6	4	7	8	TYR 9
IFH 2489	Environment	9-Jan-13	13	5	4	6	8	TYR 10
IFH 2494	Environment	21-Jan-13	8	5	4	6	8	TYR 11
IFH 2498	Environment	21-Jan-13	4	8	4	6	8	TYR 12
IFH 2500	Environment	21-Jan-13	5	7	4	6	8	TYR 13
IFH 2501	Environment	21-Jan-13	12	8	4	6	8	TYR 14
IFH 2502	Environment	21-Jan-13	11	8	4	6	8	TYR 15
IFH 2474	Raw milk	14-Dec-12	6	6	6	6	7	TYR 16
NCIMB 701790	silage	10-Oct-87	4	8	6	7	8	TYR 17
NCIMB 9582		8-Jan-65	12	7	6	6	8	TYR 18

2.4 . Design of the MLVA primers

Using the Tandem Repeat Finder program (TRF) (http://tandem.bu.edu/trf/trf.html) (Benson, 1999), we searched for targeted VNTR loci in each contig of the whole-genome draft sequence of *C. tyrobutyricum* JCM11008^T. Primers for amplifying each VNTR locus were designed using the Primer Express Software (Life Technologies, Foster City, CA). The primer was designed within 300 bp of the flanking regions of each VNTR locus.

The composition of the PCR reaction was as follows: 50.0 gl of the final mixture consisted of 5.0 gl of 10× PCR buffer, 4.0 gl of 2.5 mM dNTP mixture, 5.0 gl of each 10 gM primer (shown in Table 3), 0.25 gl (5 U/gl) of TaKaRa Taq DNA polymerase (Takara Bio Inc., Otsu, Japan), 1.0 gl of 25 ng/gl template DNA, and 29.75 gl of DW. PCR amplification was performed in a Veriti thermal cycler (Life Technologies, Foster City, CA) using the following conditions: 5 min of heating at 95 °C; 35 cycles each of 1 min at 95 °C, 30 s at the annealing temperature shown in Table 3, and 1 min at 72 °C; and 2 min at 72 °C. The PCR products were purified using the Fast Gene Gel/PCR Extraction Kit (NIPPON Genetics Co. Ltd., Tokyo, Japan). DNA sequencing was performed using the Big Dye Terminator v.3.1 Cycle Sequencing Kit (Life Technologies). After sequence analysis of the amplified VNTR loci using the 3500 Genetic Analyzer (Life Technologies), the number of repeats of the motif sequence was determined.

2.5 . Discriminatory index (DI)

Discriminatory power, i.e., the ability to distinguish between unrelated strains, was calculated based on the Simpson's index of diversity (Hunter and Gaston, 1988). As the value approaches 1, the power of the method to discriminate unrelated strains increases.