Food Microbiology 59 (2016) 213–223

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Genomic approach to studying nutritional requirements of *Clostridium tyrobutyricum* and other Clostridia causing late blowing defects

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ARTICLE INFO

Article history:
Received 5 October 2015
Received in revised form
29 April 2016
Accepted 23 May 2016
Available online 26 May 2016

Keywords:
Clostridium
Cheese
Late blowing
Vitamins
Amino acids

ABSTRACT

Clostridium tyrobutyricum is the main microorganism responsible for the late blowing defect in hard and semi-hard cheeses, causing considerable economic losses to the cheese industry. Deeper knowledge of the metabolic requirements of this microorganism can lead to the development of more effective control approaches. In this work, the amino acids and B vitamins essential for sustaining the growth of *C. tyrobutyricum* were investigated using a genomic approach. As the first step, the genomes of four *C. tyrobutyricum* strains were analyzed for the presence of genes putatively involved in the biosynthesis of amino acids and B vitamins. Metabolic pathways could be reconstructed for all amino acids and B vitamins with the exception of biotin (vitamin B7) and folate (vitamin B9). The biotin pathway was missing the enzyme amino-7-oxononanoate synthase that catalyzes the condensation of pimeloyl-ACP and L-alanine to 8-amino-7-oxononanoate. In the folate pathway, the missing genes were those coding for *para*-aminobenzoate synthase and aminodeoxychorismate lyase enzymes. These enzymes are responsible for the conversion of chorismate into *para*-aminobenzoate (PABA). Two *C. tyrobutyircum* strains whose genome was analyzed *in silico* as well as other 10 strains isolated from cheese were tested in liquid media to confirm these observations. 11 strains showed growth in a defined liquid medium containing biotin and PABA after 6–8 days of incubation. No strain showed growth when only one or none of these compounds were added, confirming the observations obtained *in silico*. Furthermore, the genome analysis was extended to genomes of single strains of other *Clostridium* species potentially causing late blowing, namely *Clostridium beijerinckii*, *Clostridium sporogenes* and *Clostridium butyricum*. Only the biotin biosynthesis pathway was incomplete for *C. butyricum* and *C. beijerincki*. In contrast, *C. sporogenes* showed missing enzymes in biosynthesis pathways of several amino acids as well as biotin, folate, and cobalamin (vitamin B12). These observations agree with the results of growth experiments of these species in liquid media reported in the literature. The results of this study suggest that biotin and folate are potential targets for reducing late blowing in cheese and highlight the usefulness of genomic analysis for identifying essential nutrients in bacteria.

1. Introduction

Late blowing refers to one of the most important fermentation defects observed in hard and semi-hard cheeses. It is characterized by the formation of butyric acid, carbon dioxide, and hydrogen during the maturation of cheese, leading to loss of its commercial value (reviewed by Bachmann, 1999; Doyle et al., 2015). The main cause of late blowing is *Clostridium tyrobutyricum*, a spore-forming, obligate anaerobic bacterium. *Clostridium* spores, present naturally in soil and at high concentrations in silage, survive the passage through the intestines of cows and accumulate in feces. The contamination of milk with these spores occurs primarily during milking. Spores survive pasteurization and *Clostridium tyrobutyricum* then outgrows in cheese during maturation. As few as 50 spores per liter of milk can induce the development of late blowing (Klijn et al., 1995; Bachmann, 1999; Doyle et al., 2015). Other Clostridia that can cause late blowing, although in milder form, are *Clostridium beijerinckii*, and *Clostridium sporogenes*. *Clostridium*

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http://dx.doi.org/10.1016/j.fm.2016.05.013

butyricum has been detected from cheeses with late blowing symptoms, however its role in cheese spoilage is still unclear (Cocolin et al., 2004; Gómez-Torres et al., 2015). In addition to good farm-management practices aiming to avoid the contamination of milk with *Clostridium* spores, several strategies have been developed to minimize late blowing (Doyle et al., 2015). Elimination of spores from milk can be achieved by bactofugation or microfiltration. However, these physical treatments require expensive equipment and change the composition of milk (Ávila et al., 2014). Alternatively, chemical methods include the use of nitrates to inhibit spore germination or lysozyme to hydrolyze linkages in the cell wall (Wasserfall and Teuber, 1979; Ávila et al., 2014). Issues concerning these practices include the risk of formation of carcinogenic compounds and allergic reactions (Fremont et al., 1997; EFSA, 2010). Moreover, bacteriocins such as nisin are not often used in Switzerland owing to lack of acceptance from manufacturers and consumers (Broughton et al., 1996; Switzerland Cheese Marketing AG, 2015). Therefore, the development of new control strategies with minimal impact on cheese quality and safety is desirable. A conceivable approach is the limitation of essential nutrients important for the growth of Clostridia. This can be achieved, for example, by using suitable starter and non-starter lactic acid bacteria that consume these nutrients directly in cheese. *Clostridium tyrobutyricum* grows in cheese using lactate and acetate as carbon sources (Ivy and Wiedman, 2014). However, the high concentrations of these compounds in cheese prevent their use as potential targets. Hence, attention should be focused on other nutrients such as amino acids and B vitamins. Unfortunately, the information available on amino acids and B vitamins essential to sustain the growth of *C. tyrobutyricum* and other late blowing–causing Clostridia is scarce. The only work reporting the growth of *C. tyrobutyricum* in a defined medium is that of Tidswell et al. (1991). In this study, a single strain involved in the industrial reduction of ketones was grown in a medium containing biotin and para-aminobenzoate (PABA). This is in agreement with the general observation that saccharolytic *Clostridium* species require biotin for growth and a few species of this group require PABA as well (Ljungdahl et al., 1989). However, the nutritional requirements of *C. tyrobutyricum* need to be confirmed conclusively using several strains, including those isolated from dairy products. Regarding the other late blowing–causing species, *C. butyricum* has been shown to require only biotin for growth (Cummins and Johnson, 1971; Himmi et al., 1999). In contrast, studies conducted using several strains of *C. sporogenes* indicated that this species requires biotin and PABA, as well as several amino acids to grow (Kindler et al., 1956; Lovitt et al., 1987). Attempts to cultivate *C. beijerinckii* in defined media supplemented with several amino acids and B vitamins have failed (Cummins and Johnson, 1971; Vos et al., 2011).

The determination of essential nutrients to sustain the growth of a microorganism can be a tedious process involving the preparation of growth media containing different combination of compounds. In the last decade, sequencing technologies with high throughput and low cost have been developed. These, together with automatic annotation tools, have made it possible to quickly sequence and analyze entire genomes of microorganisms (Heard et al., 2010; Edwards and Holt, 2013). Among the many new opportunities, the identification of essential nutrients can be accelerated by investigating the presence of the genes involved in various biosynthetic pathways. Examples in the literature where genome analysis led to the development of growth media include those involving *Lactobacillus plantarum* or *Campylobacter jejuni* (Teusink et al., 2005; Alazzam et al., 2011). Moreover, this *in silico* approach not only provides an accurate overview of the metabolic requirements of a particular organism but also highlights the genetic causes behind them.

This study aimed to determine the amino acids and B vitamins essential for the growth of *C. tyrobutyricum* through a comprehensive genomic analysis followed by experiments in defined liquid media. The *in silico* analysis was also extended to other late blowing–causing *Clostridium* species, namely *C. butyricum*, *C. beijerinckii*, and *C. sporogenes*.

2. Materials and methods

2.1. Reconstruction of vitamin and amino acid biosynthesis pathways through genome analysis

2.1.1. Bacterial genomes

Reconstruction of vitamin and amino acid biosynthesis pathways was performed for the genomes of four *Clostridium tyrobutyricum* strains and was extended subsequently to the genomes of single strains of *Clostridium butyricum*, *Clostridium beijerinckii*, and *Clostridium sporogenes* retrieved from the GenBank database. Basic information about these genomes is listed in Table 1.

2.1.2. Genome analysis

Clostridium genomes were analyzed for the presence of putative genes coding for the enzymes involved in amino acid and B vitamin biosynthesis using the following strategy. As the first step, all necessary information about amino acid and B vitamin biosynthesis pathways was collected from KEGG maps (Kanehisa and Goto, 2000; Kanehisa et al., 2014) and the literature listed in Table S1.1 and S1.2 in Supplementary File 1. A theoretical metabolic pathway was proposed for each vitamin and amino acid, which served as template in the subsequent analysis. These biosynthesis pathways are shown in Supplementary Files 2 and 3 for amino acids and B vitamins, respectively. The seven *Clostridium* spp. genomes were then submitted to RAST (Rapid Annotations using Subsystems Technology) for gene annotation (Aziz et al., 2008). The annotated genomes were searched for genes coding for the enzymes involved in the amino acid and B vitamin biosynthesis pathways using the "KEGG metabolic analysis" tool in SEED Viewer (Overbeek et al., 2005). In the case of more than one candidate for a particular reaction, a single gene was selected. In the case that a gene could not be identified using the SEED Viewer, its presence was investigated further using the Local BLAST (Basic Local Alignment Search Tool) function in CLC Workbench version 6.0.2 (CLC Bio). For this purpose, translated amino acid sequences of homologous genes belonging to *Escherichia coli* and other *Clostridium* species were retrieved from GenBank and used as templates. The GenBank accession numbers of these protein sequences are listed in Tables S4.1 and S4.2 in Supplementary File 4. Finally, the translated protein sequence of each identified gene was checked for completeness using the protein BLAST function of National Center for Biotechnology Information (NCBI) (McGinnis and Madden, 2004).

Nucleotides and translated amino acid sequences of all putative genes identified in *C. tyrobutyricum* FAM22552 and FAM22553, *C. beijerinckii* G117, *C. butyricum* DSM10702, and *C. sprogogenes* PA3679 are listed in Supplementary Files 2 and 3 for amino acids and B vitamins, respectively. To confirm the results obtained with RAST, a second annotation of the genomes was performed using Prokka (ver. 1.11; Seeman, 2014). A fasta file containing all the sequences of translated genes annotated with Prokka for the genome of *C. tyrobutyricum* FAM22552 was then blasted using CLC Workbench against the amino acids and B vitamins biosynthesis genes identified with the strategy described above for this genome. The presence of orthologous genes in the other *Clostridium* genomes was then assessed by constructing orthologous gene clusters using OrthoMCL (version 2.0.9; Li et al., 2003). OrthoMCL was also used to