J. Dairy Sci. 101:1–7
https://doi.org/10.3168/jds.2017-13372

Short communication: Growth of dairy isolates of *Geobacillus thermoglucosidans* in skim milk depends on lactose degradation products supplied by *Anoxybacillus flavithermus* as secondary species

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ABSTRACT

Thermophilic bacilli such as *Anoxybacillus* and *Geobacillus* are important contaminants in dairy powder products. Remarkably, one of the common contaminants, *Geobacillus thermoglucosidans*, showed poor growth in skim milk, whereas significant growth of *G. thermoglucosidans* was observed in the presence of an *Anoxybacillus flavithermus* dairy isolate. In the present study, we investigated the underlying reason for this growth dependence of *G. thermoglucosidans*. Whole-genome sequences of 4 *A. flavithermus* strains and 4 *G. thermoglucosidans* strains were acquired, with special attention given to carbohydrate utilization clusters and proteolytic enzymes. Focusing on traits relevant for dairy environments, comparative genomic analysis revealed that all *G. thermoglucosidans* strains lacked the genes necessary for lactose transport and metabolism, showed poor growth in skim milk, and produced white colonies on X-gal plates, indicating the lack of β-galactosidase activity. The *A. flavithermus* isolates scored positive in these tests, consistent with the presence of a putative lactose utilization gene cluster. All tested isolates from both species showed proteolytic activity on milk plate count agar plates. Adding glucose or galactose to liquid skim milk supported growth of *G. thermoglucosidans* isolates, in line with the presence of the respective monosaccharide utilization gene clusters in the genomes. Analysis by HPLC of *A. flavithermus* TNO-09.006 culture filtrate indicated that the previously described growth dependence of *G. thermoglucosidans* in skim milk was based on the supply of glucose and galactose by *A. flavithermus* TNO-09.006.
Key words: thermoresistant spore, thermophile, symbiosis

Short Communication

Heat-resistant spores from thermophilic bacilli are a major concern in dairy powder processing facilities, posing a risk for product contamination. This group of bacteria is able to grow in areas of skim milk manufacturing plants, such as heat exchangers and evaporation sections, where elevated temperatures, typically between 40°C to 65°C, are applied (Seale et al., 2015). Although these bacilli are not pathogenic, their presence as spores in end products may lead to quality issues in the respective reconstituted products when conditions are favorable for germination and outgrowth (Setlow and Johnson, 2013; Watterson et al., 2014; Wells-Bennik et al., 2016). Obviously, such quality issues could also lead to severe economic losses.

Spore-forming bacteria survive through their innate ability to resist adverse conditions in dairy manufacturing processes, including heat, mechanical disruption, and a wide variety of chemicals (Burgess et al., 2010). Moreover, both spores and vegetative cells can attach to stainless steel and fouled surfaces in dairy processing lines. Once attached to the surface, the spores may germinate, grow out, and form biofilms. Spores from thermophilic bacilli detected in end products might originate from biofilms formed in dairy processing lines (Seale et al., 2015). Thermophilic bacteria generally have a high growth rate with generation times typically in the range of 15 to 20 min under optimal conditions. Thus, high cell counts can be reached in a short period. Additionally, the resistance of the endospores to heat and chemicals makes it difficult to fully eliminate thermophilic bacteria from dairy processing environments (Burgess et al., 2010; Wells-Bennik et al., 2016). Understanding the proliferation and survival of spore-forming bacteria within dairy processing environments and dairy products is therefore a prerequisite to develop effective methods to control and reduce contamination.

Thermophilic bacteria predominantly isolated from the dairy processing industry are of the species *An-*

Received June 21, 2017.
Accepted September 20, 2017.
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oxybacillus flavithermus and the genus *Geobacillus* spp., formerly classified as *Bacillus* (Burgess et al., 2010; Zhao et al., 2013; Sadiq et al., 2016). *Anoxybacillus flavithermus* typically dominate the preheating section of the skim milk powder process, whereas a mix of *A. flavithermus* and *Geobacillus* spp. is found in the evaporation and drying stages of the process. *Geobacillus* spp. are predominantly isolated from fouling sites (Zhao et al., 2013).

To proliferate in skim milk, bacteria must utilize carbon and nitrogen sources in skim milk. Raw milk is a nutrient-rich source supporting bacterial growth. It contains approximately 3.4% protein, 3.7% fat, 4.6% lactose, and 0.7% ash by weight (Jensen, 1995). However, *G. thermoglucosidans* strains TNO-09.020 and TNO-09.023, previously isolated from a fouling site in the dairy processing pipeline, showed poor growth on skim milk plates and in liquid skim milk. This growth deficiency and their biofilm-forming capacity in skim milk were restored when *A. flavithermus*, a proteolytic thermophilic sporeformer from the microbiota of the same dairy concentrate processing plant, was added as a secondary species (Zhao et al., 2013). To understand the mechanism of growth dependence of *G. thermoglucosidans* on the presence of *A. flavithermus* as secondary species in skim milk, we performed a comparative genomic analysis on isolates of both species. In addition to the 5 dairy isolates (*Anoxybacillus flavithermus* TNO-09.006, TNO-09.014, TNO-09.016; *Geobacillus thermoglucosidans* TNO-09.020, TNO-09.023), 1 *A. flavithermus* and 2 *G. thermoglucosidans* hot spring isolates (*Anoxybacillus flavithermus* WK1, *Geobacillus thermoglucosidans* C56-YS93, *Geobacillus thermoglucosidans* Y4-1MC1) available in public genome databases were included in the current study (Table 1). Particularly, the presence and absence of genes involved in metabolic pathways including nitrogen (proteolytic system) and carbohydrate metabolism, required for growth in skim milk, were taken into consideration. Genes present in only 1 of the 2 species were enumerated and their annotated function was inspected for possible roles in growth in skim milk. Moreover, growth experiments in skim milk without and with added supplements that could conceivably support the outgrowth of *G. thermoglucosidans* were conducted. Finally, the cell-free culture filtrate of *A. flavithermus* was analyzed to identify compounds produced by this organism that could support the growth of *G. thermoglucosidans* in skim milk.

The genomes of the 8 thermophilic bacilli of the species *G. thermoglucosidans* and *A. flavithermus* (Table 1) were compared with each other and to the reference nonthermophilic strain *Bacillus subtilis* 168. For the newly sequenced genomes from this study (Zhao et al., 2012; Caspers et al., 2013, 2016), after assem-

Table 1. Presence (1) or absence (0) of genes for lactose metabolism in selected *Anoxybacillus flavithermus* and *Geobacillus thermoglucosidans* strains

Strain	NCBI accession number and reference[1]	Origin	Gene: Lactose ABC transporter, permease protein	Gene: Lactose ABC transporter, lactose-binding protein	Gene: β-Galactosidase (EC 3.2.1.23)
A. flavithermus TNO-09.006	AMCM00000000	Standard milk used for dairy processing plant, the Netherlands	1	1	1
A. flavithermus TNO-09.014	LUFB00000000	Standard milk used for dairy processing plant, the Netherlands	1	1	1
A. flavithermus TNO-09.016	LUCQ00000000	Standard milk used for dairy processing plant, the Netherlands	1	1	1
A. flavithermus WK1	GCA_000019045.1	Hot spring, New Zealand	0	0	0
G. thermoglucosidans TNO-09.020	NZ_CM001483	Casein pipe fouling in the dairy processing plant, the Netherlands	0	0	0
G. thermoglucosidans TNO-09.023	LUCT00000000	Casein pipe fouling in the dairy processing plant, the Netherlands	0	0	0
G. thermoglucosidans C56_YS93	NC_015660.1	Obsidian hot spring, Yellowstone National Park, United States	0	0	0
G. thermoglucosidans Y4_1MC1	NC_014650.1	Bath hot spring, Yellowstone National Park, United States	0	0	0

[1]National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/).