International Scholars Journals

International Journal of Microbiology Research and Reviews ISSN 2329-9800, Vol. 13 (1), pp. 001-011, May, 2025. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Through Laboratory Domestication, Clostridium Botulinum Group II Strain Beluga Exhibits Genomic and Phenotypic Diversity

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Accepted 12 May, 2025

The genetic and physiological alterations of organisms obtained through several in vitro passages lead to laboratory domestication. Higher organisms and microorganisms have both been shown to exhibit this phenomena. We examined several spore stocks of Clostridium botulinum Group II Beluga from our collection in an attempt to comprehend the effects of laboratory domestication on the foodborne pathogen and associated microbial food safety research, since that is a commonly used model strain that has been used in labs for decades. Using phenotypic microarrays, an acquired nutritional auxotrophy was verified as thymidine dependency. Parallel to this, whole-genome re-sequencing of all stocks showed that the auxotrophic stocks had a mutation in thyA, which codes for thymidylate synthase, which is necessary for the de-novo synthesis of dTMP from dUMP. By successfully introducing an intact version of thyA into a thyA-deficient Beluga variant stock, thymidine prototrophy was recovered, demonstrating that the thymidine auxotrophy was only caused by an SNP in thyA. According to our data, this mutation has been present and sustained in laboratory stocks for almost 30 years. It is harmful when growth circumstances are inadequate in terms of nutrients and in a chemically defined media. However, since obtaining the strain, the mutation has remained unknown, most likely as a result of routinely using culture conditions that are best suited for growth performance. This study highlights the necessity of closely observing model strains that are frequently utilized in lab settings at the phenotypic and genomic levels. Compromised strains have the potential to produce inaccurate predictions in applications such as food safety challenge testing, which could have detrimental effects. We advise avoiding single-colony passaging and maintaining low passage numbers of laboratory strains to reduce the chance of acquiring mutations. Furthermore, to rule out DNA mutations that could compromise the integrity and repeatability of study data, pertinent strains should undergo routine WGS analyses and physiological validation.

Key words: Foodborne pathogen, Laboratory strain, Genetic stability, Adaptation, Whole genome sequencing,

Botulism, Challenge study, Clostridium botulinum.

INTRODUCTION

As an adaptive reaction to the chosen conditions, organisms that are repeatedly cultivated in a controlled environment may have genetic and phenotypic changes (Eydallin et al., 2014). This phenomenon, which is frequently referred to as "laboratory domestication" (Eydallin et al., 2014), has been documented in higher organisms such as the fruit fly Drosophila melanogaster (Stanley and Kulathinal, 2016) and the nematode Caenorhabditis elegans (Sterken et al., 2015), as well as microorganisms frequently used in biomedical research, such as Bacillus subtilis (McLoon et al., 2011) and Escherichia coli (Eydallin et al., 2014; Liu et al., 2017) as well as Bacillus subtilis (McLoon et al., 2011). According to

Zeder (2015), domestication is defined as "a sustained multigenerational, mutualistic relationship in which one organism assumes a significant degree of influence over the reproduction and care of another organism […] frequently increasing the fitness of both the domesticator and the target domesticate." The repeated cultivation of microorganisms (the target domesticate) by humans, the domesticator, in processes such as food fermentations, which have been carried out for centuries and industrialized in the last century, is an example of domestication leading to an increase in microbial fitness (Steensels et al., 2019). On the other hand, microbial domestication may also lead to undesirable phenotypic changes, such as decreased organism fitness in harsh environments or compromised biofilm formation (McLoon et al., 2011). Crucially, some modifications may go unnoticed in most situations but have a significant impact on the use of microorganisms, for example, in research. Laboratory domestication of bacteria has been thoroughly investigated in E. coli (Eydallin et al., 2014; Liu et al., 2017) and B. subtilis (Barreto et al., 2020; McLoon et al., 2011).

Domesticated bacteria differ genetically from their wild counterparts due to untargeted mutagenesis, which was used to explore biochemical processes and create isolates capable of introducing genetic material (McLoon et al., 2011; Zeigler et al., 2008). Additionally, it has been reported that natural bacterial isolates can undergo fast spontaneous genetic modifications after being transferred from their limited natural habitat into a laboratory where they are grown under conditions that are optimized for growth in terms of temperature, nutrients, and other variables (Barreto et al., 2020; Liu et al., 2017). Two of four natural E. coli strains had mutations in the DNA mismatch repair gene mutL as a result of prolonged stationary phase cultivation, which subjected the bacterium to nutritional stress (Liu et al., 2017). These isolates are therefore more likely to undergo mutations, which could lead to improved nutritional stress adaption at the expense of decreased genetic stability. Remarkably, all four strains' descendant isolates had mutations in rpoS, a gene essential for the stress response, when they were subcultured more frequently without nutritional stress (Liu et al., 2017). This suggests that under laboratory circumstances designed to observe microbial growth, the stress response—which is crucial for survival in wild bacterial habitats—plays a negligible role in bacterial fitness. Because the bacterium can survive and inherit genetic modifications that would be harmful in its natural habitat, it is reasonable to assume that other features that were established during bacterial evolution but are not necessary in laboratory settings may also be lost or changed.

Research on general microbial metabolism and the contribution of particular nutritional and metabolic variables to bacterial virulence and disease is made possible by the use of minimum and chemically defined media. The extremely powerful botulinum neurotoxin (BoNT), which causes the paralytic illness known as botulism, is produced by the food-borne bacteria Clostridium botulinum. BoNT production was already associated with environmental factors such as temperature, acidity, and nutrient availability (Bonventre and Kempe, 1959a; Holdeman and Smith, 1965; Kindler et al., 1956) in the early days of C. botulinum research (Bonventre and Kempe, 1959b; Tanner and Oglesby, 1936). More recent studies have refined these associations on a mechanistic level (Popoff and Brüggemann, 2022). We tried to cultivate this strain from our routine stock (referred to here as FI-2003) in a nutrient-poor, chemically defined medium in order to investigate the potential effects of nutrients on the physiology of the non-proteolytic C. botulinum Group II strain Beluga E (producing BoNT/E1), which is frequently used in our and many other laboratories (Whitmer and Johnson, 1988). Unlike the original article (Whitmer and Johnson, 1988), Beluga's growth was not supported by the medium. Beluga could not be successfully cultivated in our first attempt to increase the nutrient complexity of the medium by substituting casamino acids and tryptophan for single amino acid components. In contrast, other non-proteolytic Group II strains from our collection, such as Eklund 17B, BL86/33, CB11/1-1, and BL93/8, grew easily in the semi-defined medium based on casamino acids. We tested several Beluga stocks that were kept in our lab and found that one of them could grow in the semi-defined medium while the others couldn't. Characterizing the various observable phenotypes of our several Beluga populations in nutrient-poor medium and linking these variations to genetic changes was the study's goal. To do this, we used whole-genome sequencing with phenotypic microarrays to find a potential nutritional auxotrophy. The goal of this work is to draw attention to the possibility that unintended spontaneous genetic mutations could lead to the inadvertent domestication of microbes in biomedical research. If this happens, it could jeopardize the quality and reproducibility of research findings.

2. Materials and methods

2.1. Bacterial strains and culturing conditions

Stocks of C. botulinum Group II strain Beluga E characterized in this study are listed in Table 1. In our laboratory, the stocks were stored as spore stocks in sterile distilled water and routinely grown in anaerobic nutrient-rich tryptone-peptone-glucose-yeast-extract (TPGY) medium in an anaerobic workstation (MG1000 anaerobic workstation; Don Whitley Scientific Ltd., Shipley, UK) under anaerobic atmosphere of 85 % N2, 10% CO2, and 5 % H2 at 30 ◦C. The TPGY medium consisted of 50 g/l tryptone, 5 g/l peptone, 20 g/l yeast extract (Difco, BD Diagnostic Systems, Sparks, MD, USA), 4 g/l D-glucose, and 1 g/l sodium thio-glycolate. Additionally, nutrient-poor semi-defined media (SDM-II and pSDM-II) based on the minimal medium MII (Whitmer and Johnson, 1988) were used for phenotypic characterization. The minimal medium MII was supplemented with 11.89 mg/l CoCl2*6H2O and 0.173 mg/l Na2SeO3, and the single amino acid components were replaced by 1 g/l (pSDM-II) or 10 g/l (SDM-II) acid hydrolyzed casein (Millipore, Merck, Darmstadt, Germany)

and 0.01 g/l (pSDM-II) or 0.1 g/l (SDM-II) L- tryptophan. Glucose was added at 1 g/l (pSDM-II) or 10 g/l (SDM-II). Resazurin and NaHCO3 were omitted, as they were considered as not required in the original MII medium (Whitmer and Johnson, 1988). When indicated, the medium was supplemented with 100 or 500 mg/l thymidine. Unless otherwise indicated, all reagents were acquired from Sigma-Aldrich (Merck, Darmstadt, Germany). All experiments, apart from the thymidine auxotrophy identification (Section 2.2), were con- ducted in triplicate using three biological replicates.

2.2. Identification of thymidine auxotrophy

Using Biolog Phenotypic MicroArray (PM) plates PM1, 3B, 4A, 7, and 8, potential nutritional auxotrophies of C. botulinum Beluga stock FI-2003 were identified (Biolog, Hayward, CA, USA). SDM-II was used in place of the media that came with the PMs. To guarantee the best possible dissolution of test substrates, 50 µl of SDM-II was pre-filled into each PM well. The plates were then anaerobically packed and kept for two days at 4 ◦C.

The producer's website, https://www.biolog.com/wp-content/uploads/2023/12/00A-042-Rev- D-Phenotype-MicroArrays-1-10-Plate-Maps.pdf, contains the substrates used in the arrays. The Beluga stock was re-inoculated into new SDM-II (1:100) after being grown overnight from spores in TPGY in two successive passes. 50 µl of the SDM-II culture was applied to each PM plate well right after inoculation. After 24 and 48 hours, the plates were visually examined for growth after being incubated anaerobically at 30 °C.

2.3. Functional confirmation of thymidine auxotrophy

Three separate passages of the C. botulinum strain Beluga stocks FI-2003 (thymidine auxotroph) and FI-1985 (thymidine prototroph) were cultured overnight from spores in TPGY. 50 ml of pSDM-II medium was inoculated with cells from 1 ml of the second overnight cultures, either with or without supplementation of 100 or 500 mg/l thymidine and TPGY in a 1:100 ratio. The cells were then rinsed with 1 ml of pSDM-II medium. A 96-well plate was filled with three 300-µl aliquots of each replicate, which were then cultured anaerobically for five days at 30 °C in a Hidex Sense Multimodal Microplate Reader (Hidex, Turku, Finland). The change in optical density at 595 nm (OD595) was used to measure growth. As previously mentioned, the most likely number of spores was calculated from the 50-ml cultures after 48 and 120 hours of growth (Nowakowska et al., 2022).

2.4. Growth curve analysis

Growth curves were created using the cultures' OD595 measurements in order to describe the development of several C. botulinum Beluga stocks in various mediums. Using the open source program AMiGA, which models microbial growth curves using Gaussian process regression, we examined the maximum culture cell density (ODmax) and the overall growth over the observed time (area under the curve AUClin and log-transformed area under the curve AUClog) (Midani et al., 2021).

2.5. Quantification of botulinum neurotoxin

Using anti-bodies generously supplied by Brigitte and Martin Dorner, Robert Koch Institute, Berlin, the production of botulinum neurotoxins from 1 ml of culture after 24 hours of growth was measured using BoNT/E sandwich ELISA, as previously described (Nowakowska et al., 2022).

2.6. Whole genome sequencing, read mapping, SNP calling, and variant filtering

The Wizard® Genomic DNA Purification Kit (Promega, WI, USA) was used to isolate the total genomic DNA of C. botulinum Beluga stocks FI-1985, FI-1994, FI-1996, FI-2003, FI-2008, FI-2016a, and FI-2016b (Table 1) in accordance with the manufacturer's instructions. Before being sent for sequencing, DNA was measured using NanoDrop (Thermo Scientific, MA, USA). Whole genome sequencing (WGS) was performed on the isolates' genomic DNA at the Institute for Molecular Medicine Finland (FIMM, University of Helsinki, Finland). The Nextera DNA Flex Library Prep Kit (Illumina, CA, USA) was used to prepare the Illumina sequencing library, and BluePippin (Sage Science, MA, USA) was used to further select DNA fragments that ranged in size from 500 to 850 bp. The Illumina HiSeq2500 platform was used to sequence the genome. As previously mentioned (Douillard et al., 2022), paired-end raw readings for each C. botulinum Beluga stock were imported into CLC Genomics Workbench v 11.0.1 (Qiagen GmbH, Germany), trimmed, and mapped against the genome of C. botulinum Beluga (NZ_ACSC01000000, here US-2009, Table 1). In the CLC Genomics Work-bench, SNP, InDel, and structural variant calling were also performed. Beluga isolates UK-0187 and UK-0603, which were previously sequenced and deposited in the NCBI Sequence Read Archive under the respective SRR8981336 and SRR8981597, were used in a similar analysis. Supplementary Data file S1 describes the parameters and analytic workflow of the CLC Genomics Workbench.

2.7. Genetic complementation

The clostridial shuttle vector pMTL82151 (Heap et al., 2009) was used to complement the defective thyA gene in C. botulinum Beluga stock FI-2003 as described earlier (Kirk et al., 2012). Genomic DNA isolated from stock FI-1985 served as template to PCR-amplify a 1146-bp

fragment consisting of thyA and the regions 266 bp upstream and 78 bp downstream of the coding sequence using primers 5′-TATA-TAGCGGCCGCGTTTACTCCACCTTTCTTTTTTC-3′ and 5′-AGCAGCCA-

TATGCTTAAGTTCAATTACTTTGCCTAG-3′. The amplicon was purified,

double-digested with NotI/NdeI, and ligated into the plasmid

pMTL82151. The plasmid pMTL82151::thyA was cloned in chemically competent E. coli NEB 5α cells (New England Biolabs, Inc., Ipswich, MA, USA) and its sequence was confirmed by Sanger sequencing. The plasmid was transformed into E. coli CA434 and conjugated into C. botulinum Beluga stock FI-2003.

To confirm the genetic complementation using pMTL82151::thyA, the Beluga FI-2003 pMTL82151::thyA stock was grown in pSDM-II, without or with 100 mg/l thymidine supplementation. All media were supplemented with 15 μg/ml thiamphenicol to ensure plasmid propagation.

2.8. Statistical analysis

Following an F-test for equality of variance in Microsoft Excel, two-tailed Student's t-tests were conducted to look for significant differences in growth, spore generation, and neurotoxic production between the C. botulinum Beluga stocks and various media.

2.9. Data accession

The NCBI received the raw sequencing data under the BioProject number PRJNA976516. Supplementary Table S1 contains the Biosample and Sequence Read Archive numbers. The Institute of Food Research (UK) provided samples UK-0187 and UK-0603, which were obtained under BioProject number PRJNA517184.

3. Results

3.1. C. botulinum Beluga stock FI-2003 is auxotrophic for thymidine

For C. botulinum Beluga stock FI-2003, auxotrophy for thymidine was discovered utilizing nutrient-poor growing media (SDM-II) in conjunction with the Biolog PM system. Only in wells containing thymidine (plates PM1 and PM3B), thymine (plate PM3B), thymidine-3′-monophosphate (plate PM4A), or thymidine-5′-monophosphate (plate PM4A) did FI-2003 grow in PMs within 24 hours into visually detectable cultures; no discernible growth was found in the other wells of the same PMs or in PM7 and PM8, suggesting that stock FI-2003 was auxotrophic for thymidine.

3.2. Thymidine auxotrophy could be functionally complemented

To determine if thymidine supplementation would completely restore the phenotype of Beluga stock FI-2003 in comparison to FI-1985, nutrient poor medium pSDM-II was either used alone or supplemented with two distinct amounts of thymidine (100 or 500 mg/l) or TPGY. As was first noted, in pSDM-II, stock FI-2003 did not develop in the absence of thymidine supplementation. Nevertheless, thymidine administration at both doses facilitated stock FI-2003 development in pSDM-II in a concentration-dependent manner (Fig. 1). While growth was hindered with lower thymidine concentrations, FI-2003 and FI-1985 were able to grow similarly in terms of cell density and growth rate when supplemented with 500 mg/l of thymidine (Fig. 1). In TPGY, both stocks saw identical growth (data not shown).

3.3. Effect of thymidine supplementation on BoNT production

The synthesis of BoNT appeared to be concentration dependent for FI-2003: the concentration of BoNT was nearly two times ($p < 0.01$) greater at 500 mg/l thymidine than at 100 mg/l (Fig. 2A).

In thymidine-supplemented pSDM-II, the stock FI-2003 generated lower BoNT concentrations than FI-1985 during the course of 24 hours of development ($p < 0.01$, Fig. 2A), while TPGY showed no difference. However, under the majority of tested conditions, no discernible difference between the two tested Beluga stocks was seen when BoNT production was linked to the microbial growth metrics AUClin, AUClog, and ODmax (Fig. 2B, C, and D).

BoNT/ODmax was lower ($p < 0.05$) in the cultures supplemented with 500 mg/l pSDM-II compared to 100 mg/l pSDM-II for both FI-2003 and FI-1985 (Fig. 2D). Additionally, when the cultures were grown in 500 mg/l of thymidine as opposed to 100 mg/l, BoNT/AUClin and BoNT/AUClog were lower (Fig. 2B and C), indicating a detrimental effect of high thymidine levels on BoNT production under growth circumstances with low nutritional levels.

3.4. Heat resistant spores

In every medium that allowed for development, the spore counts of the thymidine-auxotrophic stock FI-2003 were often greater than those of the corresponding FI-1985 cultures (Fig. 3). The pSDM-II treated with 500 mg/l thymidine had the highest spore concentrations for both stocks, surpassing even the nutrient-rich TPGY. The information points to a beneficial, dose-dependent effect of thymidine supplementation on sporulation.

3.5. A SNP in thyA was detected in several Beluga E stocks

In every medium that allowed for development, the spore counts of the thymidine-auxotrophic stock FI-2003 were often greater than those of the corresponding FI-1985 cultures (Fig. 3). The pSDM-II treated with 500 mg/l thymidine had the highest spore concentrations for both stocks, surpassing even the nutrient-rich TPGY. The information points to a beneficial, dose-dependent effect of thymidine supplementation on sporulation.

The predicted active site of ThyA (UniProt ID B2TR56_CLOBB) changed from cysteine to phenylalanine at amino acid position 143 after a guanine base on the coding strand at position 61865 was swapped out for a thymine base in all thyA-deficient stocks. In comparison to the thyA-intact stocks (31 to 38 SNPS and InDels), the Beluga E stocks with the disclosed thyA mutation also had a greater overall number of genetic alterations (65 to 77 SNPS and InDels compared to the US-2009 genome) (Fig. S1). Only the thyA-deficient strains possessed 33 SNPs, some of which resulted in missense mutations, such as in the DNA glycosylase gene clo_3407. Table S2 lists every genetic variant found in the examined genomes in comparison to the US-2009 reference sequence.

3.6. thyA mutation preserved in Beluga stocks since at least 1994

We conducted variant analysis (Douillard et al., 2022) to determine the genetic relatedness of the sequenced Beluga populations by comparing them to the publicly available Beluga genome reference sequence US-2009 and two additional publicly available Beluga E sequences, UK-0187 and UK-0603. All of our thyA-deficient Beluga E stocks are most likely descended from FI-1994, a stock that has been propagated in our lab since 1994, according to the analysis. Stock FI-1985, which was obtained from IFR, carried an intact thyA and was, predictably, closely related to the IFR stocks UK-0187 and UK-0603 (Fig. 4A and B).

3.7. thyA mutation can be genetically complemented

We employed plasmid pMTL82151:: thyA to genetically complement stock FI-2003 with an intact thyA from stock FI-1985 in order to verify that the detected thyA mutation was, in fact, the origin of the observed thymidine auxotrophy (Heap et al., 2009).

Without thymidine supplementation, stock FI-2003 was able to grow in pSDM-II thanks to the thyA complementation (Fig. 5A). However, the development of FI-2003 pMTL82151::thyA in pSDM-II was feeble and the phenotypic was not entirely recovered. This could be because of the antibiotic marker present in the nutrient-poor growth medium or the plasmid's metabolic burden. Although FI-2003 pMTL82151::thyA grew more when 100 mg/l thymidine was added to pSDM-II (Fig. 5B), the culture density was less than that of FI-1985. However, the complementation confirms our theory that a mutation in thyA caused some of our Beluga spore stocks to be unable to grow in chemically specified conditions.

4. Discussion

Here, we describe the use of phenotypic microarrays in conjunction with nutrient-poor growing medium to detect acquired thymidine auxotrophy in a C. botulinum Group II Beluga stock. When regularly cultivated in nutrient-rich growth media, this strain has been used extensively in our study and has never shown any abnormalities in its growth behavior, BoNT production, or sporulation. Only after failed attempts to cultivate one of our spore stocks (FI-2003) on a chemically defined medium that was reported to favor the growth of strain Beluga (Whitmer and Johnson, 1988) did the detected nutrient auxrophy become apparent. We believe that this auxotrophy's tolerance results from domestication in the lab, as has been documented in other model organisms (Liu et al., 2017; McLoon et al., 2011).

A number of Beluga stocks from our collection were tested in nutrient-poor medium (pSDM-II) without thymidine supplementation following the unsuccessful cultivation of FI-2003 in nutrient-poor medium. Only the stock FI-1985 (received in 1985 from the Institute of Food Research, now Quadram Institute, Norwich, UK) showed signs of growth. All thymidine-auxotrophic Beluga stocks had an SNP in the gene thyA, which codes for the thymidylate synthase ThyA, according to a comparative genomic analysis of our Beluga stocks and the genomes of the publicly available genomes. In contrast, the stock FI-1985 had an intact thyA. The thymidine auxotrophy of FI-2003 was restored by genetic complementation with an intact thyA. The pyrimidine deoxyribonucleotide biosynthesis pathway step of dUMP conversion into dTMP (=thymidylate) was disrupted and enzymatic activity was probably lost as a result of the discovered SNP's amino acid ex-change from cysteine to phenylalanine in the anticipated active region of ThyA (Fig. 6). dTMP is necessary for DNA synthesis since it is the precursor of thymine. Most species have an alternate dTMP synthesis system because deoxyribonucleotide synthesis is essential for supplying the building blocks needed for DNA replication. Thymidine kinase in C. botulinum Beluga may use a another mechanism to transform thymidine into dTMP (Tdk, Fig. 6). The growth of stock FI-2003 in thymidine-supplemented pSDM-II indicates that this thymidine-dependent pathway spontaneously compensates the defective thyA under appropriate environmental growth conditions. In our laboratory, where the strains are regularly cultivated in nutrient-rich TPGY that provides high thymidine concentrations, this probably allowed the thyA-deficient C.

botulinum Beluga stocks to survive, hence avoiding detection of the mutation. However, loss of ThyA function appears to be harmful in environments where thymidine is sparse, such as in nutrient-poor media.

Reduced levels of BoNT production, the most significant virulence factor of C. botulinum, were the result of the thyA mutant stock's growth being compromised under harsh conditions.

Our data, however, show that the thyA mutation has no direct effect on the synthesis of BoNT. Mouse lethality doses per culture volume or BoNT concentration are commonly used to quantify the toxicity of C. botulinum cultures. However, when evaluating various media or nutrients, or in intervention studies, for instance, BoNT levels should also be connected to the bacterial growth of the culture under study, particularly in growth-modulating circumstances. Unfortunately, the C. botulinum research community has not yet established a standard procedure for doing so. Here, we connected the measured total BoNT concentrations to a number of growth characteristics, taking into account the overall growth over a 24-hour period, which is indicated by the culture's highest optical density (ODmax) and area under the curve (AUClin, AUClog). This method showed that the observed variations in total BoNT concentrations between the two stocks' cultures were probably due to variations in bacterial growth rather than the mutation's effect on BoNT production per se.

Our findings imply that the creation of BoNT may be impacted by thymidine itself. When compared to 100 mg/l of thymidine-supplemented pSDM-II, the BoNT/AUC and BoNT/ODmax for stock FI-2003 cultures were marginally lower when grown in 500 mg/l (Fig. 2B–D). A comparable pattern was found for FI-1985. These results might suggest that adding a lot of thymidine to a medium that is low in nutrients inhibits the production of boNTs in response to growth. As a survival strategy, C. botulinum cells may favor sporulation (explained below) over excessive BoNT synthesis in a nutrient-poor environment. Although it is yet unclear what elements influence the decision-making process in the synthesis of BoNT, the availability of DNA building blocks in an otherwise limited environment may be a factor.

It's interesting to note that ThyA increases virulence in a number of harmful bacteria (Kok et al., 2001; Kriegeskorte et al., 2014). Mutants of Salmonella typhi-murium thyA exhibited reduced in vivo pathogenicity in mice and were unable to develop intracellularly in vitro in a human epithelial cell line or a macrophage-like cell line (Kok et al., 2001). Natural thyA mutations were frequently found in trimethoprim-sulfamethoxazole (SXT) resistant strains of Staphylococcus aureus that were isolated from individuals with cystic fibrosis following prolonged antibiotic treatment (Chatterjee et al., 2008; Kahl et al., 2005). The pyrimidine deoxyribonucleotide production pathway is inhibited by SXT. Therefore, it is likely that greater SXT resistance and long-term adaptation to SXT treatment are conferred by loss of ThyA function and the easily active and efficient use of the altered dTMP synthesis pathway. ThyA has been discussed as a genetic marker for the most virulent strains of Clostridium difficile and linked to "hypervirulence" (Knetsch et al., 2011; Robinson et al., 2014). ThyA was specific for ribotype 027 strains, sometimes known as hypervirulent strains, while screening 88 distinct C. difficile strains. In contrast, non-ribotype 027 strains have an alternative thymidylate synthase gene, thyX (Robinson et al., 2014). ThyA can speed up DNA replication and has a greater catabolic efficiency than ThyX (Escartin et al., 2008). Because the thyA gene variation increases the development and fitness of C. difficile isolates, it can therefore be viewed as a competitive advantage. Whether thyA mutations result in decreased virulence of C. botulinum is still up for investigation. Although toxicoinfectious botulism (Artin et al., 2007; Lúquez et al., 2010), a disease form of botulism that shares etiological similarities with C. difficile infection, is unlikely to be caused by C. botulinum type E, intestinal botulism caused by C. botulinum Group I strains is relatively common in infants and adults with underlying conditions. In an infant botulism model, it would be intriguing to investigate if a thyA mutation hinders colonization or competition with the gut flora.

Although C. botulinum is commonly regarded as a foodborne pathogen and an environmental bacterium, little is known about its natural habitat. Beluga and other strains of Group II type E are frequently identified in fish and marine and freshwater sediments in temperate aquatic habitats (Huss, 1980; Hyytia et al., 1998), and fish and other seafood are frequently associated with related foodborne illnesses (Lindstro¨m et al., 2006; Peck, 2009). Despite being viewed as a weak rival, C. botulinum is likely to have a growth advantage in limited natural settings due to its highly effective thyA encoded version of thymidylate synthetase and a functional pyrimidine metabolism-based dTMP production pathway. Loss of functional ThyA would probably be counter-selected since it could have negative effects and reduce fitness. However, tolerance to the identified SNP in thyA and persistence in several stocks cultivated and sporulated from the thyA defective ancestor FI-1994 that we acquired in 1994 have been made possible by repeated cultivation under favorable laboratory growth circumstances. This thymidine auxotrophy, which does not change the phenotypic under our standard laboratory culture conditions, was first discovered in 2020 during our unsuccessful attempts to cultivate the FI-2003 stock in a nutrient-poor medium. The lack of the SNP in stock FI-85 or published genomes (US-2009, UK-0187, and UK-0603) indicates that the mutation was acquired either prior to entering our lab or during the initial stages of our lab. One could argue that this conserved mutation is an undesirable byproduct of domestication in a lab.

Compared to the corresponding FI-1985 cultures, the thyA-deficient stock FI-2003 sporulated at higher levels. Laboratory domestication of the "offspring" stocks may have led to selection for a sporulation efficient phenotype through repeated reproduction of spore stocks from the original thyA-deficient Beluga E strain (FI-1994). Naturally, the greater amounts of sporulation and/or spore germination compared to FI-1985 may have been caused by other changes found in the FI-2003 genome. Genetic variations in membrane-associated, metabolic, regulatory, or hypothetical

genes as well as non-coding regions (Suppl. Table S2/Fig. S1) may influence sporulation through as-yet-unidentified mechanisms, despite the fact that our genome analysis did not identify any clear targets in the known sporulation or germination system that would support this assumption. Additionally, in relation to FI-2003 and US-2009, an SNP in a potential spore-cortex-lytic enzyme gene (clo_2115) was found in the FI-1985 genome. Potentially compromised function of this enzyme, which is crucial for effective spore cortex hydrolysis during germination (Chirakkal et al., 2002), may reduce stock FI-1985's germination efficiency and make it more difficult to detect dormant spores in assays that primarily depend on viable, germinating spores.

According to our research, thymidine may have an effect on C. botulinum spore development. Both tested stocks, FI-2003 and FI-1985, produced more spores in pSDM-II supplemented with 500 mg/l of thymidine than in pSDM-II supplied with 100 mg/l of thymidine and in comparison to nutrient-rich TPGY. Given the need of DNA synthesis for chromosome replication in sporulation, thymidine supplementation may actually promote sporulation in pSDM-II under the demanding starving circumstances encountered during growth. Although the exact beginning and signaling of sporulation in C. botulinum are yet unknown, ambient nutrition availability is anticipated to be a major factor in clostridial sporulation (Shen et al., 2019). More research is necessary to determine how thymidine as a nutrition affects sporulation in C. botulinum.

Compared to the thyA-intact Beluga stocks, a greater total number of genetic alterations were found in the thyA-deficient Beluga stocks. Furthermore, compared to the thyA-intact samples, a higher percentage of these mutations were found in coding areas. Under particular culture conditions, the identified mutations may have resulted in phenotypic variations since they affected genes that coded for metabolic enzymes, transcriptional regulators, and membrane and transport proteins. An intriguing SNP was discovered in a gene producing DNA glycosylase, an enzyme involved in base excision repair, that was uniquely shared by all thyA-deficient strains. This enzyme's impairment may make the stocks less genetically stable (Jacobs and Scha¨r, 2012) and result in the greater mutation frequency we saw in the strains lacking thyA.

Unintentionally selecting for mutations that result in an undesirable phenotype that is undetectable under typical laboratory culture conditions is a risk associated with each passage through a single colony. Under strict conditions in a particular study design, these mutations may still manifest as phenotypes. Food safety may be compromised if strains with reduced virulence are unintentionally used in challenge tests or predictive model development to assess microbiological food safety. This could result in an underestimating of the proliferation and toxin production of foodborne pathogens. Therefore, to lower the risk of laboratory domestication, the utilization of clonal spores or frozen stocks of bacterial cells generated from single colonies randomly chosen from nutrient-rich agar plates should be reexamined. The issue may be lessened by limiting the number of passes and subculturing cell mass in addition to regular genetic monitoring. To continuously improve optimal laboratory procedures, more research is necessary.

5. Conclusion

In our laboratory's stocks of C. botulinum Group II strain Beluga E, we discovered an acquired thymidine auxotrophy. An SNP in the thyA gene, which codes for the enzyme thymidylate synthetase, which is required for the de-novo synthesis of dTMP, a crucial DNA building block, was the source of the auxotrophy. The common practice of cultivating in nutrient-rich media likely obscured the phenotype, which is why this mutation, which affects the microbial phenotype only under strict nutrient conditions but not in nutrient-rich routine media, was maintained for decades in several ancestral spore stocks in our collection. This observation is seen as an illustration of undesirable laboratory domestication. Since thyA has been linked to virulence and fitness in a number of microbial pathogens (Kok et al., 2001; Kriegeskorte et al., 2014; Robinson et al., 2014), its mutation may render thyA-deficient C. botulinum stocks unsuitable for use in studies pertaining to virulence and food safety. Our results emphasize how crucial it is to thoroughly characterize the bacterial isolates and strains chosen for study. To prevent unintentional selection of potentially harmful mutations, we advise against repeatedly and single-colony passaging laboratory strains. Finding mutations that might affect the strains' phenotype and, consequently, the results of research experiments and the interpretation of findings seems to require routine whole genome re-sequencing and phenotypic validation of laboratory strains.

Declaration of Generative AI and AI-assisted technologies in the writing process

The writers utilized ChatGPT to enhance some language while preparing this work. The authors took full responsibility for the publication's content after utilizing this tool/service, reviewing and editing it as necessary.

Declaration of competing interest

None.

Data availability

Raw sequencing data were deposited in NCBI under the BioProject number PRJNA976516

Acknowledgements

For their outstanding technical support, the authors thank Erika Pitka¨nen, Esa Penttinen, and Hanna Korpunen. We are grateful to Prof. Nigel Minton for supplying the complementation plasmid system and to our collaborators Dr. Martin and Prof. Brigitte Dorner for graciously donating the ELISA antibodies and procedure.

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Table 1
Clostridium botulinum Group II wild-type strain Beluga E stocks.

Name	Year of spore stock preparation	Source	References
FI-2003	2003	University of Helsinki, Finland	This work
FI-1985	1985	Institute of Food Research (now Quadram Institute), UK	This work
FI-1994	1994	University of Helsinki, Finland	This work
FI-1996	1996	University of Helsinki, Finland	This work
FI-2008	2008	University of Helsinki, Finland	This work
FI-2016a	2016	University of Helsinki, Finland	This work
FI-2016b	2016	University of Helsinki, Finland	This work
US-2009	n.a.[a]	Los Alamos National Laboratory, USA	NZ_ACSC01000000
UK-0187	n.a.	Institute of Food Research (now Quadram Institute), UK	Brunt et al., 2020
UK-0603	n.a.	Institute of Food Research (now Quadram Institute), UK	Brunt et al., 2020

[a] n.a. = not available.

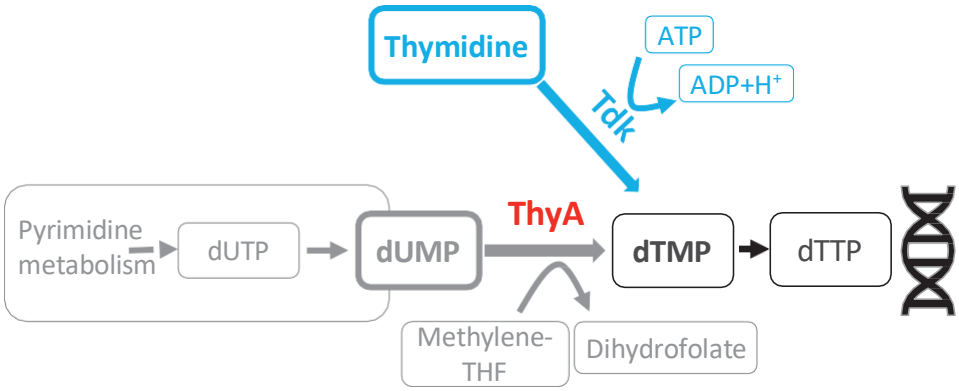

Fig. 6. Simplified pyrimidine metabolism pathway. Grey: impaired pathway due to *thyA* gene disruption, blue alternative dTMP synthesis pathway utiliz- ing thymidine.