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Published in:
F E M S Microbiology Letters

Link to article, DOI:
10.1093/femsle/fnw126

Publication date:
2016

Document Version
Publisher's PDF, also known as Version of record

FEMS Microbiology Letters, 363, 2016, fnw126

doi: 10.1093/femsle/fnw126
Advance Access Publication Date: 11 May 2016
Research Letter

RESEARCH LETTER – Physiology & Biochemistry

The global regulator CodY is required for the fitness of *Bacillus cereus* in various laboratory media and certain beverages

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One sentence summary: Deletion of the global regulator coding CodY gene in *B. cereus* ATCC 14579 differentially affects fitness in various laboratory media and beverages.

Editor: Ezio Ricca

ABSTRACT

The impact of gene mutations on the growth of the cells can be studied using pure cultures. However, the importance of certain proteins and pathways can be also examined via co-culturing wild type and its mutant derivative. Here, the relative fitness of a mutant strain that lacks the global nitrogen regulator, CodY, was examined in *Bacillus cereus*, a food poisoning Gram-positive bacterium. Fitness measurements revealed that the Δ*codY* strain was outcompeted when cocultured with the wild-type ATCC 14579 under various rich laboratory medium, and also when inoculated in certain beverages. In nutrient-poor minimal medium, the Δ*codY* mutant had comparable fitness to the wild-type strain. Interestingly, the relative fitness of the Δ*codY* strain was antagonistic when it was cultivated in apple or orange juices due to unknown properties of these beverages, highlighting the importance of chemical composition of the test medium during the bacterial fitness measurements.

Keywords: *Bacillus cereus*; fitness; nitrogen metabolism; CodY; biofilm

INTRODUCTION

To understand how microbes adapt to certain conditions, we need to understand in addition to the regulatory cascades that respond to a certain stress also the genetic background that is evolved and conserved to be able to respond to the environmental changes. Such a genetic adaptation might also result in gene loss if a certain gene is not required for the fitness of the microbe (Dufresne, Garczarek and Partensky 2005; Morris, Lenski and Zinser 2012). Genetic adaptations are driven by successive process of fixing beneficial mutation, and it is driven by natural selection (Kassen 2014). Such a selection acts not only on genes coding for proteins with structural, enzymatic or similar roles, but also on transcriptional regulators that modify the expression of genes from the previous category. Conserved regulators found in wide variety of microorganisms are supposed to carry an evolutionary advantage for the cells, and therefore, the loss of these components is selected against. *Bacillus cereus* is a member of the genus *Bacillus* and normally isolated from the soil (Stabb, Jacobson and Handelsman 1994; Garbeva, van Veen and van Elsas 2003), while it is also frequently associated with food poisoning and spoilage (Ehling-Schulz, Frenzel and Gohar 2015). *Bacillus cereus* is believed to exist in versatile environment ranging from the soil as saprophyte to insect or mammal host as potential pathogen (Jensen *et al.* 2003), where it encounters various environmental conditions and survives different stress conditions (van Schaik and Abee 2005; Abee *et al.* 2011; Mols and Abee 2011a,b). Interestingly, genome analysis of

Received: 29 January 2016; **Accepted:** 9 May 2016

the *B. cereus* group suggested that this group has adapted to protein-rich diet rather than carbohydrate-based diet preferred by the saprophytic *B. subtilis* (Garbeva, van Veen and van Elsas 2003). *Bacillus cereus* is a food poisoning microorganism (Ehling-Schulz, Frenzel and Gohar 2015; Gopal *et al.* 2015) and has been associated with fatal cases of infections (Veysseyre *et al.* 2015). *Bacillus cereus* has been reported to survive industrial pasteurization processes in the form of spores and psychrophilic strains, which are able to cope with refrigeration temperature, resulting in a potential danger for the dairy and food industry (Griffiths 1992). Therefore, the applicability of various antimicrobial compounds against *B. cereus* has been examined in different fruit juices and other food products (Grande *et al.* 2005, 2006, 2007; de Oliveira Junior *et al.* 2015).

To efficiently survive variable environmental conditions and to adapt to changes in nitrogen resources, low G+C Gram-positive bacteria have acquired a conserved global regulator called CodY. In *Bacilli*, CodY responds to the intracellular levels of guanosine triphosphate (GTP) and branched chain amino acids that enhance dimerization and DNA binding, respectively (Ratnayake-Lecamwasam *et al.* 2001; Shivers and Sonenshein 2004; Handke, Shivers and Sonenshein 2008). In the presence of these cofactors, CodY represses the transcription of numerous genes (Molle *et al.* 2003). The target genes code for proteases, amino acid transporters and catabolic pathway (Sonenshein 2005), but other processes are also affected indirectly by CodY in *Bacilli*, including sporulation, competence for DNA uptake, motility and biofilm formation (Serror and Sonenshein 1996; Molle *et al.* 2003; Rasko *et al.* 2005; Sonenshein 2005). Furthermore, CodY plays an important role in the regulation of virulence factors in pathogenic bacteria, such as *B. anthracis*, *Clostridium difficile*, *Listeria monocytogenes*, *Staphylococcus aureus* and *Streptococci* (Stabb, Jacobson and Handelsman 1994).

Previous studies on the CodY regulator of *B. cereus* strains revealed its importance in the production of toxins in addition to regulating the transcription of genes related to nitrogen metabolism (Frenzel *et al.* 2012; Lindbäck *et al.* 2012). In addition, whole-genome transcriptome analysis also showed that other phenotypes, e.g. motility and biofilm formation, are also differentially affected by the deletion of the *codY* gene in *B. cereus* (Lindbäck *et al.* 2012). Similar to other Gram-positive pathogens, CodY also influences virulence gene expression in *B. cereus* (Frenzel *et al.* 2012; Lindbäck *et al.* 2012), via modulating the uptake of the signal peptide of PlcR, the major virulence transcriptional regulator (Slamti *et al.* 2016). Interestingly, the growth properties of *B. cereus* ATCC 14579, on different media, were found to be similar between the wild-type strain and its Δ*codY* variant, and only minor delay in growth of Δ*codY* derivative was found in BHI medium (Lindbäck *et al.* 2012). However, a shift of mid-logarithmic phase cells from nutrient-rich to minimal medium caused a major delay in the growth of the Δ*codY* mutant compared to the wild-type strain (Lindbäck *et al.* 2012). Nevertheless, generating a Δ*codY* strain in *B. thuringiensis* strain 407 was only possible in the presence of *codY* gene in trans (Slamti *et al.* 2016), suggesting that deletion of the *codY* gene in certain strains might result suppressor mutations elsewhere in the genome similar to *Streptococcus pneumoniae* (Caymaris *et al.* 2010). In addition, the impact of gene deletions might differ depending on the strain used, as observed for the trait of biofilm development of *B. cereus* (Hsueh, Somers and Wong 2008; Lindbäck *et al.* 2012).

The sociomicrobiology of the *B. cereus* group was studied only recently (Raymond and Bonsall 2013). Recent publications described various traits of *B. thuringiensis* that are benefiting virulence (Garbutt *et al.* 2011; Raymond *et al.* 2012; Zhou *et al.* 2014; Deng *et al.* 2015), including the importance of spatial structure, division of labor, impact of strain diversity or cooperation during virulence. These studies clearly show that the relative fitness of certain mutant strains can be most efficiently determined in competition to a common strain (i.e. compared to the wild type used in this study) (Raymond and Bonsall 2013).

The purpose of this work was to examine if the presence of this conserved global regulator enhance the fitness of the bacterium, *B. cereus* ATCC 14579 in various laboratory media and certain beverages. Competition experiments revealed that a *codY* mutant has lower fitness under most of the tested laboratory media and beverages, which might explain why the global regulator CodY is conserved in these bacteria.

MATERIALS AND METHODS

Strains and media

Bacillus cereus ATCC 14579 and its marker-less Δ*codY* derivative (Lindbäck *et al.* 2012) was used in this study. The following growth media were used for cultivation: LB medium (Lennox broth, Carl Roth, Germany; 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, pH 7.0), BHI medium (Brain-Heart-Infusion Broth, Carl Roth Germany; 7.5 g/L Calf Brain Infusion, 10 g/L Beef Heart Infusion, 10 g/L Peptone, 2 g/L Glucose, 5 g/L NaCl, 2.5 g/L Na_2HPO_4, pH 7.4), CSE medium (chemically defined minimal medium; 4 g/L KH_2PO_4, 16 g/L $K_2HPO_4{\cdot}3H_2O$, 3.3 g/L $(NH_4)_2SO_4$, 8 g/L K-glutamate, 6 g/L Na-succinate, 5 g/L glucose, 2.32 mg/L $MnSO_4{\cdot}4H_2O$, 0.123 g/L $MgSO_4{\cdot}7H_2O$, 50 mg/L tryptophane, 22 mg/L NH_4Fe(III)-citrate) and EPS medium (biofilm medium; 7 g/L K_2HPO_4, 3 g/L KH_2PO_4, 0.1 g/L NaCl, 0.125 g/L yeast-extract, 0.1 g/L $MgSO_4{\cdot}7H_2O$, 0.01 g/L $CaCl_2$, 1 mg/L $FeSO_4$, 1 g/L glucose). Milk was prepared by dissolving 10 g skim milk powder (Carl Roth, Germany) in 100 ml water and autoclaved at 110 °C for 7 min. Commercial orange and apple juices (distributed by Eckes-Granini (Nieder-Olm, Germany)) were used after adjusting the pH to 6.0–6.2 with NaOH as low pH of the medium inhibits the growth of *B. cereus*. Haemolysis positive or negative colonies were detected on BHI agar plates (1.5% agar) supplemented with 4% defibrinated sheep blood (Fiebig-Nährstofftechnik, Germany). All growth experiments were performed at 30 °C.

PCR validation of *codY* mutation

The identity of wild-type and Δ*codY* colonies after selected competition experiments was verified by PCR. Colonies with or without haemolytic halo were picked from BHI blood agar plates; PCR was directly performed on the cells using oligonucleotides oTB92 (5′-GTGCAACGTGACATTCTG-3′) and oTB93 (5′-CACAAGCATCTTCTATCC-3′) in the presence of DreamTaq DNA polymerase (Thermo Fisher Scientific, Germany). The PCR resulted 1424 and 657 bp products on the wild-type and Δ*codY* strains, respectively.

Growth properties

Optical densities (OD) of cultures were recorded in various media after 1:100 dilutions of overnight cultures. For rich media, 150 μL aliquots of the cultures were pipetted into a 96-well plate, and optical density was followed in a TECAN plate reader (at least six biological replicates with four technical replicates each, >24 samples for each strains and media). For minimal medium, strains were cultivated in 100 mL bottles to reduce aggregation,

and OD was recorded every hour using a UV 1600-PC spectrophotometer (VWR, Darmstadt, Germany).

Competition assays

The relative fitness of the Δ*codY* strain was determined in the presence of wild-type strain. Strains were grown in LB medium for 16 h at 30 °C at 225 rpm, and their colony forming units were determined by plating on BHI agar medium. Wild-type and mutant strains were mixed in 10:1, 1:1 and 1:10 ratios and diluted 1:100 in certain medium. After 16 h of growth at 30 °C 225 rpm, a dilution series was plated on BHI blood agar medium to calculate the colony forming units of wild-type (haemolysis proficient) and Δ*codY* (haemolysis deficient) strains.

Biofilm formation

After preculturing in LB medium, overnight grown single strains or their co-culture (similar as for competition assays) were diluted 1:100 in EPS medium, and 1 mL culture was inoculated into one well of a 24-well plate (VWR, Germany). After 24 h at 30 °C, the medium fraction was removed and fresh EPS medium was added to the wells. Cells from the biofilm were released by mild sonication (2 × 12 pulses of 1 s with 30% amplitude; Ultrasonic Processor VCX-130, Zinsser Analytics, Frankfurt am Main, Germany), and the colony forming units were determined on BHI blood agar medium as described above.

Fitness calculations

Fitness was calculated for each competition experiments where the ratio of the Malthusian parameters (m) of each competing strain was determined (Lenski 1991). Malthusian parameter was calculated as follows: $m = \ln(N_f/N_0)$, where N_f and N_0 are the final and starting colony forming units.

RESULTS AND DISCUSSION

The growth yield of wild-type and Δ*codY* strain on various media is comparable

The growth properties of *B. cereus* wild-type (ATCC 14579) and Δ*codY* strains were previously compared on nutrient-rich and minimal media by following the optical density, but only minor delay was observed with no effect on growth yield (Lindbäck *et al.* 2012). The growth properties of wild-type and Δ*codY* strains were followed in LB, BHI and CSE media. Although a minor delay was observable in the median of the wild-type and Δ*codY* growth curves when cultivated in rich media, no significant difference was identified here. In addition, the growth yields of the wild-type and Δ*codY* strains were determined by detecting the colony forming units of pure bacterial cultures grown for 16 h. Strains were cultivated in various laboratory media and certain beverages under well-mixed conditions (shaken cultures at 225 rpm). After 16 h, the cell counts of the strains were comparable in all cultures (Fig. 1) and no significant differences were found (t-test; >0.15).

Using the crystal violet assay that reports the surface attached biomass and its surrounding matrix, it was previously shown that Δ*codY* strain formed an increased biofilm compared to the wild type (Lindbäck *et al.* 2012). Such an enhanced biofilm formation could be the result of increased cell number or superior matrix production or both. The cell count numbers of 24h-grown biofilms were determined after mild removal of the attached cells (see materials and methods). These experiments showed that the Δ*codY* biofilm contained slightly more bacterial cells compared to the wild type ($3.3{\cdot}10^7 \pm 2.2{\cdot}10^6$ and $2.5{\cdot}10^7 \pm 2.5{\cdot}10^6$, respectively; Fig. 1B, $P < 0.01$) confirming previous observations on the increased biofilm forming ability of the Δ*codY* strain.

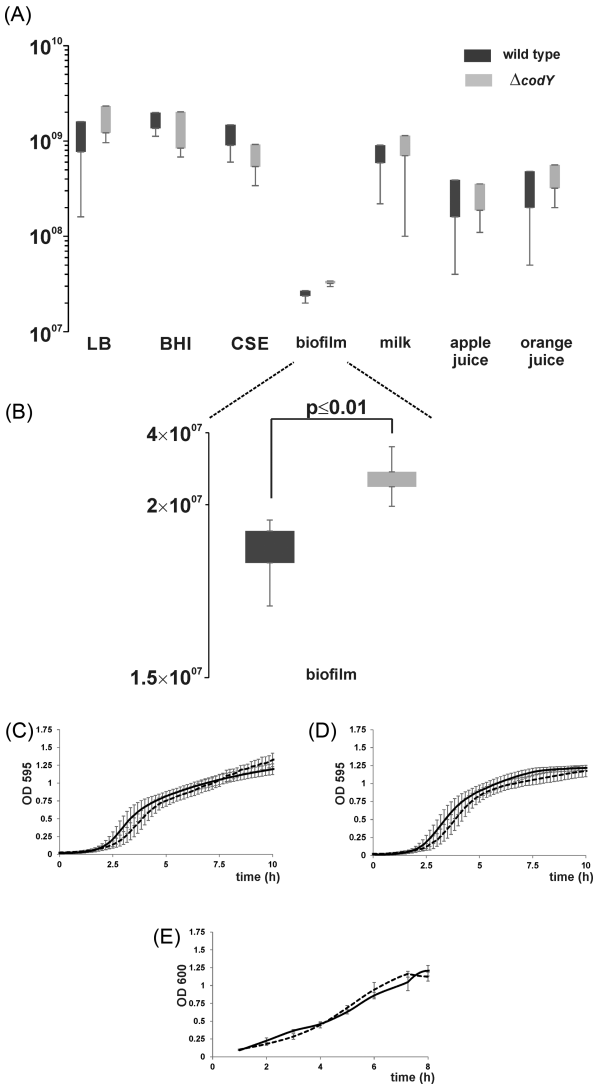

Figure 1. Growth of *B. cereus* ATCC 14789 and its *codY* derivative. Colony forming unit of bacterial cultures are shown grown for 16 h under various conditions (**A**) or 24 h in biofilms (**B**). Data present the averages of at least three independent cultures for each conditions and strains. OD of wild-type (continuous lines) and Δ*codY* (dashed lines) cultures are presented that were cultivated in LB (**C**), BHI (**D**) or CSE (**E**) media. For the cultures grown in rich media, at least six biological replicates with four technical replicates each (>24 samples for each strains and media) were used. Growth was recorded in TECAN plate reader at 595 nm. For CSE medium, OD of four biological replicate cultures were detected at 600 nm using a UV 1600-PC spectrophotometer (VWR, Darmstadt, Germany).

Fitness on various laboratory media

Recently, researchers realized that certain relevant properties of microbes can be adequately examined in competition with other species or strains (Raymond and Bonsall 2013). These studies generally use previously developed methods from the field of evolutionary biology to measure the relative or competitive

fitness of strains (Lenski 1991). Here, the relative fitness of *B. cereus* strain was examined in which the *codY* gene was deleted from the genome. When the *codY* gene is disrupted in *B. cereus*, about 250 and 180 genes are up- and downregulated, respectively, in the strain ATCC 14579, respectively (Lindbäck *et al.* 2012). This suggests that the function of CodY global regulator might be required for efficient growth on various media. However, the growth yield of wild type and its Δ*codY* derivative was found to be comparable under all examined culturing conditions except when biofilm formation was studied. Previously, we could show that certain traits that are not essential *per se* for a phenotype or differentiation pathway (e.g. motility of *B. subtilis* during biofilm formation at the air-medium interface) might be important for the full fitness of the bacterium when a mutant is competed against the wild type in a co-culture experiment (Hölscher *et al.* 2015). Therefore, the relative fitness of the Δ*codY* strain was examined during co-cultivation on various laboratory media. To distinguish the wild-type and Δ*codY* strains after co-cultivation, the differential hemolysis properties of the strains on blood agar medium were used as a marker. Certain colonies with hemolysis (wild type) and without (Δ*codY*) were selected and their genotype was confirmed using PCR specific to the *codY* region (data not shown).

When certain strains are competed at equal initial frequencies and the strains grow similarly, the relative fitness of the mutant is around 1. However, at low or high initial frequencies, the fitness might increase or decrease due to negative frequency selection (MacLean 2008). Thus during negative frequency-dependent selection, when a genotype is rare, it is relatively favored by selection and it will increase in frequency, but when a genotype is abundant, it is no longer selected for, thus it will decrease in frequency. Using a fitness presentation where the x axis depicts the initial ratio of Δ*codY*/wild-type strains (e.g. Fig. 2), this is visualized by a trend line that starts above 1 and crosses the x axis at 1 to 1 initial ratio in the case of equal fitness properties of the co-cultured strains. Competition experiments showed that the Δ*codY* strain had a reduced relative fitness in nutrient rich laboratory media (Fig. 2A-B), while in minimal medium, the relative fitness of Δ*codY* derivative is negative frequency dependent (Fig. 2C), but generally similar to the wild type.

The presented competition experiments clearly indicated that under most of the cultivating conditions, the wild-type ATCC 14579 strain harboring a functional CodY protein had higher competitiveness suggesting that CodY has an important impact on the fitness of *B. cereus* under these conditions. When the *codY* gene is removed, expression levels of CodY repressed genes are enhanced in *B. cereus* ATCC 14579 (Lindbäck *et al.* 2012), which could increase the general metabolic costs of the cells. However, the impact of such a metabolic cost can be only occasionally detected, when the mutant strain is competed with its wild-type ancestor. When *B. cereus* is grown in minimal medium, the amount of branched chain amino acids is low and the cells face starvation (i.e. low amounts of GTP), the CodY protein is not active. Under these conditions, the CodY-repressed genes are expressed similarly in the Δ*codY* mutant that is supported by the comparable relative fitness of the Δ*codY* to the wild type when they are co-cultivated in CSE minimal medium.

Fitness during biofilm formation

In several instances, secretion of so called public goods that are shared among the members of the community has a cost for the producer organism. Due to this cost, cells that are producing less public goods have an advantage. Biofilms present a fascinating system, where public good sharing and the effect of spatial distribution can be studied (Kovács 2014; van Gestel *et al.* 2014). Biofilm formation of *B. cereus* depends on various secreted polymers (Caro-Astorga *et al.* 2014; Gao *et al.* 2015). Biofilm formation of *B. cereus* is enhanced when the *codY* gene is deleted resulting in a higher amount of cells attached to the microtiter plate wells and increased crystal violet detected biomass (Lindbäck *et al.* 2012). Therefore, to observe how the wild-type ATCC 14579 and Δ*codY* strains compete under biofilm promoting conditions, the two strains were mixed at various ratio, inoculated in EPS biofilm medium and cultivated for 24 h. The relative fitness of the Δ*codY* strain was only comparable to the wild-type strain when Δ*codY* was used at low frequency, otherwise a slight reduction of fitness was observed (Fig. 3), suggesting that the two strains co-colonize the biofilm and the Δ*codY* strain might have a somewhat reduced fitness due to increased biofilm matrix production (i.e. expression of genes coding for the protein

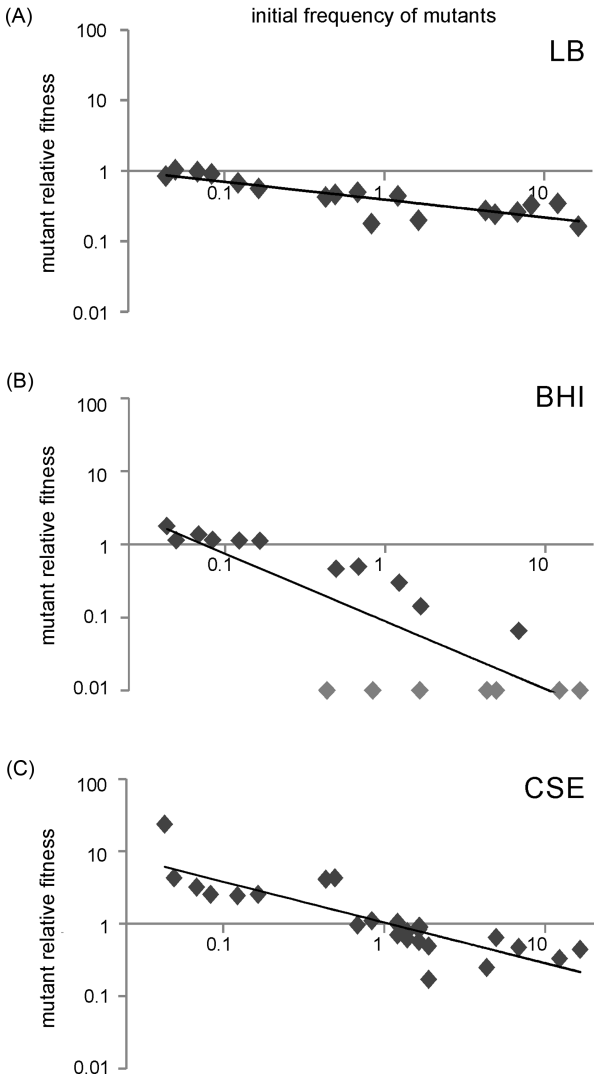

Figure 2. Relative fitness of *B. cereus codY* strain grown in LB, BHI or CSE media inoculated at various initial ratio of Δ*codY*/wild-type strains. Gray colored rhombuses indicate data points with fitness values <0.01. Increased or decreased fitness are presented by data points above or below value 1, respectively. At low or high initial frequencies, fitness values might increase or decrease due to negative frequency selection (see text for details).

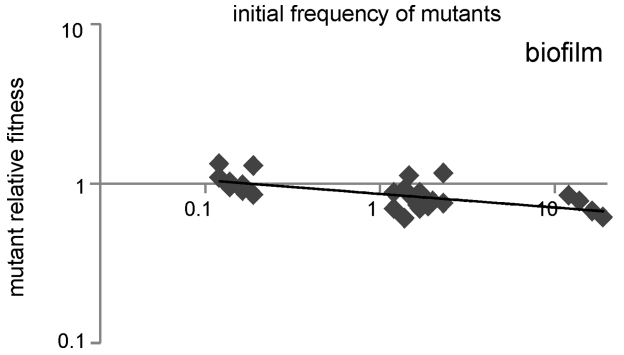

Figure 3. Relative fitness of *B. cereus codY* strain during biofilm formation inoculated at various initial ratio of Δ*codY*/wild-type strains. Increased or decreased fitness are presented by data points above or below value 1, respectively. At low or high initial frequencies, fitness values might increase or decrease due to negative frequency selection (see text for details).

component of biofilms are increased in the Δ*codY* strain (Lindbäck *et al.* 2012)).

Fitness on beverages

The above presented results suggested that wild-type *B. cereus* had growth advantage over the Δ*codY* mutant during competition under most conditions, except in minimal medium. *B. cereus* is a food poisoning microorganism, resulting in spoiled or emetic toxin containing products (Ehling-Schulz, Frenzel and Gohar 2015; Gopal *et al.* 2015). To further examine the relevance of these observations under food related conditions, the competition experiments were performed in various beverages: milk, apple and orange juices. Interestingly, the relative fitness of the Δ*codY* strain did not follow the same trend in all beverages used. The relative fitness of Δ*codY* was lower than the wild-type strain when cultivated in milk or apple juice, but had slightly increased relative fitness when grown in orange juice (Fig. 4).

The properties and the documented composition (e.g. fruit contents, fat, carbohydrate, sugar or salt levels) of the two beverages used are similar and the juices were purchased from the same supplier. It is intriguing why the Δ*codY* strain had an advantage when grown in orange juice, while the wild type was benefitted in the apple juice. As CodY alters the transcription of several hundreds of genes in *B. cereus* (Frenzel *et al.* 2012; Lindbäck *et al.* 2012), it is plausible that certain pathways important for the efficient growth in apple or orange juices are differently affected by CodY. Detailed examination of the two juices, like determination of the organic acid composition and concentration or antimicrobial compounds, might facilitate the identification of the factor(s) that differentially benefit one strain over the other.

CONCLUSION

Growth yield is generally used to estimate fitness of a microbial strain under certain cultivating conditions. The presented experiments highlight that distinct bacterial strains harboring comparable growth yield might have different fitness when competed with each other. Remarkably, *B. cereus* ATCC 14579 has a higher relative fitness compared to its Δ*codY* derivative under various laboratory media and beverages, but not when cultivated in orange juice. Therefore, to conclude whether the growth properties of certain strains are different from others necessitates competition experiments. *B. cereus* Δ*codY* mutant was

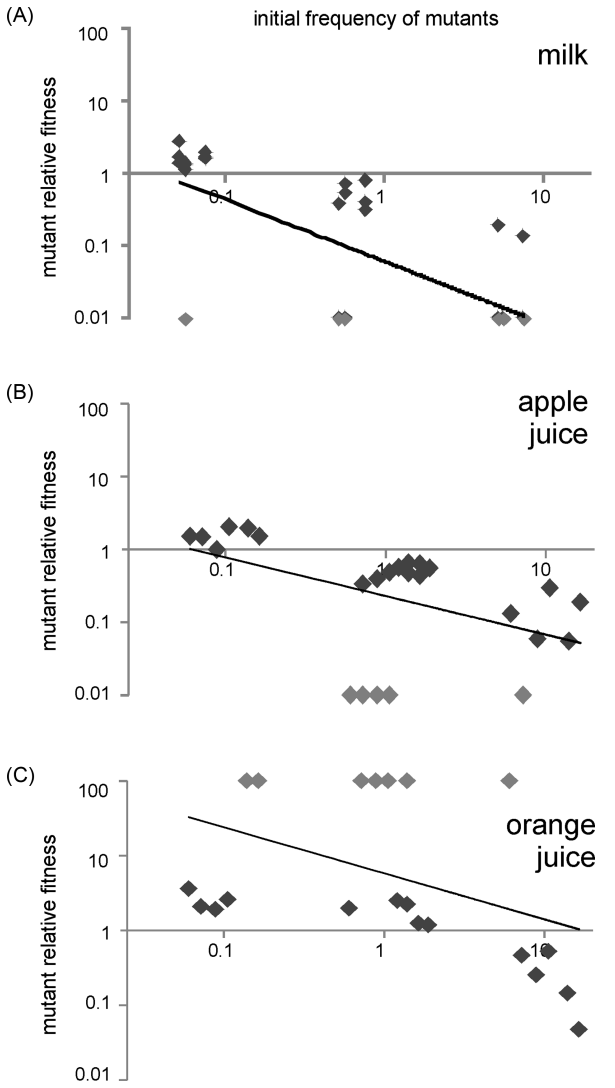

Figure 4. Relative fitness of *B. cereus codY* strain grown in milk **(A),** orange- **(B)** and apple juice **(C)** initiated at various initial ratio of Δ*codY*/wild-type strains. Gray colored rhombuses indicate data points with fitness values <0.01 or >100. Increased or decreased fitness are presented by data points above or below value 1, respectively. At low or high initial frequencies, fitness values might increase or decrease due to negative frequency selection (see text for details).

shown to produce decreased amounts of toxins (Lindbäck *et al.* 2012). Therefore, it will be interesting to examine the relative fitness of the Δ*codY B. cereus* strain in host models systems, such as *Galleria mellonella* (Ramarao, Nielsen-Leroux and Lereclus 2012). It is debatable whether the Δ*codY* mutants can exploit the toxins produced by the wild type in the host and how spatial structure defines the competitiveness of these strains, similar to *B. thuringiensis* mutants that lack quorum sensing, the density dependent cell-cell communication system (Zhou *et al.* 2014).

FUNDING

Work in the laboratory of ATK is supported by a Marie Curie Career Integration Grant (PheHetBacBiofilm), Grants KO4741/2-1 and KO4741/3-1 from the Deutsche Forschungsgemeinschaft (DFG).

Conflict of interest. None declared.

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