Food Microbiology 36 (2013) 113–122

Contents lists available at SciVerse ScienceDirect

Food Microbiology

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

Unsaturated fatty acids from food and in the growth medium improve growth of *Bacillus cereus* under cold and anaerobic conditions

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ARTICLE INFO

Article history:
Received 8 August 2012
Received in revised form
12 March 2013
Accepted 15 April 2013
Available online 9 May 2013

Keywords:
Bacillus cereus
Cold
Anaerobiosis
Membrane
Fatty acid

ABSTRACT

In a chemically defined medium and in Luria broth, cold strongly reduced maximal population density of *Bacillus cereus* ATCC 14579 in anaerobiosis and caused formation of filaments. In cooked spinach, maximal population density of *B. cereus* in anaerobiosis was the same at cold and optimal temperatures, with normal cell divisions. The lipid containing fraction of spinach, but not the hydrophilic fraction, restored growth of *B. cereus* under cold and anaerobiosis when added to the chemically defined medium. This fraction was rich in unsaturated, low melting point fatty acids. Addition of phosphatidylcholine containing unsaturated, low melting point, fatty acids similarly improved *B. cereus* anaerobic growth at cold temperature. Addition of hydrogenated phosphatidylcholine containing saturated, high melting point, fatty acids did not modify growth. Fatty acids from phospholipids, from spinach and from hydrogenated phosphatidylcholine, although normally very rare in *B. cereus*, were inserted in the bacterium membrane. Addition of phospholipids rich in unsaturated fatty acids to cold and anaerobic cultures, increased fluidity of *B. cereus* membrane lipids, to the same level as those from *B. cereus* normally cold adapted, i.e. grown aerobically at 15 °C. *B. cereus* is therefore able to use external fatty acids from foods or from the growth medium to adapt its membrane to cold temperature under anaerobiosis, and to recover the maximal population density achieved at optimal temperature.

1. Introduction

Bacillus cereus is a Gram-positive spore-forming foodborne pathogen able to survive cooking or the mild heat treatments applied to many processed foods. Such foods are usually kept at low temperature and packaged under vacuum or modified atmospheres. These conditions considerably reduce growth of *B. cereus* in laboratory media (i.e. the chemically defined medium MOD and LB) (de Sarrau et al., 2012). Cold alone slows growth but does not influence biomass production (Pandiani et al., 2011). Anaerobiosis reduces biomass production (i.e. maximal population density) (Rosenfeld et al., 2005; Zigha et al., 2007), and this reduction is strongly amplified by the synergistic action of anaerobiosis and cold (de Sarrau et al., 2012). Indeed, under anaerobiosis, biomass was considerably lower at low temperature than at optimal temperature. In addition, cells division was impaired, resulting in long filaments (de Sarrau et al., 2013). This was shown in laboratory media and should be tested in a real food.

In Europe, cooked vegetables represent an important range of refrigerated products, often packaged under anaerobiosis, frequently contaminated with, and supporting growth of, *B. cereus* (Bae et al., 2012; Carlin et al., 2000; Choma et al., 2000; Guinebretiere et al., 2001; Samapundo et al., 2011). Among vegetables, we used cooked spinach as an important product of some food companies. Spinach represents a complex matrix, containing for 100 g of fresh weight, 2.65 g of protein, 0.3 g of fat in majority phospholipids, 0.61 g of carbohydrates, 2.58 g of fiber, 0.08 g of organic acids, 1.69 g of minerals, chlorophyll, secondary metabolites as phenolic compounds and carotenoids, and 91.5 g of water. Compared to laboratory media, vegetables could exert an inhibitory effect on foodborne bacteria (Babic et al., 1997; Valero and Salmerón, 2003). In contrast, some compounds in foods may help bacteria to overcome stressful condition. For instance, compatible solutes, not present in chemically defined media, are necessary for

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http://dx.doi.org/10.1016/j.fm.2013.04.008

the adaptation of *Listeria monocytogenes* to osmotic stress (Sleator et al., 2003). In the case of cold under anaerobiosis, growth of *B. cereus* is presumably inhibited because membrane lipids and membrane fluidity can not be adapted to low temperatures without oxygen (de Sarrau et al., 2012). Presence of lipids in foods could therefore be an important factor in *B. cereus* adaptation to these conditions. Brinster et al. (2009) showed that *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Enterococcus faecalis* and *Enterococcus faecium* are able to insert fatty acids from human serum or Tween 80 in their membrane in presence of an inhibitor of fatty acid biosynthesis or after deletion of the genes involved in fatty acid biosynthesis. However, it is not known if bacteria can use fatty acids from the growth media to compensate a defect in membrane adaptation to cold.

The purpose of this study is to test in a real food the impact of cold on anaerobic growth of *B. cereus*, considering both population increase and ability of cells to divide normally, and to investigate the possible role of lipids. Cooked spinach was used as the food system.

2. Materials and methods

2.1. Bacterial strains and media

The type strain *B. cereus*, ATCC 14579 was used. This strain belongs to phylogenetic group IV defined by Guinebretiere et al. (2008), and its genome was sequenced in 2003 (Ivanova et al., 2003). The chemically defined MOD medium (de Sarrau et al., 2012) was supplemented with 1 g l^{-1} of K_2HPO_4, 6 g l^{-1} of $(NH_4)_2SO_4$, 0.04 g l^{-1} of $MgSO_4$, 30 mM of glucose, and 0.1 M of phosphate buffer pH 7.4. Luria broth (LB) "low salt" containing 10 g l^{-1} of peptone, 5 g l^{-1} of yeast extract, 0.5 g l^{-1} of NaCl, was supplemented with 50 mM of glucose and 0.1 M of phosphate buffer pH 7.4. LB agar was made of LB without glucose and phosphate buffer to which 15 g l^{-1} agar was added. To prepare spinach medium, blanched, frozen Spinach (Auchan, France) was thawed and blended with distilled water (1 g ml^{-1} of spinach) in a stomacher. Spinach medium was tyndallized (3 treatments of 80 °C for 1 h followed each by 24 h at 30 °C) and pH was adjusted to 7.4 with phosphate buffer, final concentration 0.1 M. Laboratory media and spinach medium were reduced by heating and bubbling with nitrogen during 1 h. Hungate tubes and penicillin flasks were filled with 12 ml and 100 ml of medium, respectively, under nitrogen flow. Whenever needed, phosphatidylcholine (PC, Sigma), or hydrogenated phosphatidylcholine (HPC, Sigma) were added to the laboratory media. Polar and apolar spinach extract, prepared as described below (lipid extraction), were added to MOD in amounts equivalent to 1 mg ml^{-1} of spinach. Glucose solution, buffer, PC and HPC solutions, apolar and polar spinach extracts were sterilized by filtration at 0.2 μm and added in media. To avoid oxygen contamination, solutions added to media were reduced, and injected with syringes through a butyl septum. Cultures were inoculated with overnight anaerobic precultures at 37 °C in MOD for cultures in MOD and LB for cultures in spinach or LB, to reach an $A_{(600\ nm)}$ of 0.02 corresponding to 10^6 CFU ml^{-1}.

2.2. Morphology and viability of cells

Light microscopy examinations were carried out with a phase contrast Olympus BX 50 instrument (Rungis, France) with a BX-FLA reflected light fluorescence attachment. Pictures were taken with a coolSNAP EZ camera (Tucson, USA) and processed with Micromanager software, ImageJ, and PMC Capture Pro software for epifluorescence microscopy. Pictures of Syto 9 and propidium iodide staining, were taken separately with the corresponding filters and combined using PMC Capture Pro software.

In laboratory media, lengths of between 91 and 372 cells sampled at the beginning of the stationary phase were measured with ImageJ software, for each *B. cereus* culture. Live/dead staining was used to count the proportion of live (green) and dead cells (red). Green was obtained by staining with Syto 9 (3.34 mM) at 1.5 μl ml^{-1} of cultures (excitation at 485 nm and emission at 498 nm). Red was obtained by staining with propidium iodide (20 mM) at 1.5 μl ml^{-1} of cultures (excitation at 536 nm and emission at 617 nm). Syto 9 stains nucleic acids after membrane permeabilization. Propidium iodide stains nucleic acids of cells that have lost membrane integrity. Proportions of live and dead cells were counted on 15 pictures taken randomly in samples of cultures at the beginning of the stationary phase.

In spinach medium, 1 ml of diluted cultures was filtrated on 0.2 μm filter in Nitrocellulose (Millipore, Darmstadt, Germany), the filter was stained with 4,6-diamino-2-phenylindole (DAPI, Sigma, Saint Louis, USA) and blue fluorescent bacterial cells were counted against the background of spinach particles using coolSNAP ez camera. To evaluate the number of cells in 1 ml we calculated that one filter corresponded to 70802 camera fields, so one field corresponds to 0.0141 μl of filtered culture. We counted at least 10 cells for each replicate.

2.3. Growth parameters

Growth in MOD and LB was monitored in Hungate tubes at 15 and 37 °C shacked at 200 rpm until stationary phase (at least 3 points at the same $A_{(600\ nm)}$ value). Growth was followed by measurement of attenuance at 600 nm ($A_{600\ nm}$) recorded directly in Hungate tubes with a spectrophotometer (Helios Epsilon, Thermo Scientific). Growth in flasks was monitored during 10 days at 15 °C, overnight at 37 °C, with shaking at 200 rpm. Growth in spinach was followed both by CFU counts on LB agar and counting cells by epifluorescence microscopy as described above.

2.4. Glucose consumption

A sample of culture was taken at the beginning of stationary phase. The aliquots were centrifuged at 7000 *g* for 5 min at 4 °C, and the supernatants were stored at −20 °C until analysis. Glucose, was measured using Biosentec enzymatic kits (Toulouse, France), following the manufacturers' instructions. Two technical replicates were done for each three biological sample.

2.5. Lipid extraction

Cultures were centrifuged (7500 *g* for 10 min at growth temperature) and supernatants were discarded. Pellets were suspended in 5 ml of MOD and centrifuged (7500 *g* for 10 min at growth temperature). This step was repeated at least 3 times to wash the cells. To obtain between 10 and 20 mg of lipids, we pooled 4 or 5 flasks for culture at 15 °C under nitrogen alone or with HPC, and 2 flasks for cultures with PC. Pellets were kept at −20 °C.

Extractions were performed as described by Bligh and Dyer (1959). Pellets were suspended in 500 μl of phosphate buffer 0.1 M at pH 7.4. Then, 3.75 ml of a 1:2 chloroform-methanol mixture were added, and the samples were shaken vigorously and left at room temperature for 1 h. Then, 1.25 ml of chloroform was added, followed by 1.25 ml of phosphate buffer at 0.1 M at pH 7.4. To separate phases, samples were centrifuged at 1000 *g* for 5 min at room temperature. Organic phases were transferred to a clean tube and dried by nitrogen flow and vacuum desiccation for 1 h. Dry lipids were kept at −20 °C under nitrogen to avoid oxidation. The same extraction was used to separate polar phase and apolar phase of spinach. 100 g of spinach were incubate 2 h at