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RESEARCH ARTICLE

The long-term survival of *Propionibacterium freudenreichii* in a context of nutrient shortage.

Short running head: *P. freudenreichii* survival

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Abstract

Aims: *Propionibacterium freudenreichii* is an actinobacterium widely used in dairy industry during the ripening process of Swiss-type cheeses and which presents probiotic properties. PF is reportedly a hardy bacterium, able to survive during the cheese-making process and when subjected to digestive stresses. During this study the long-term survival of *P. freudenreichii* was investigated for 11 days by means of phenotypic characterization in a culture medium without the addition of any nutrients.

Methods and Results: For 11 days, in a non-nutrient supplemented culture medium, eight strains were monitored by measuring their optical density, counting colony-forming units (CFU) and using LIVE/DEAD staining and microscopy observation. Under these conditions, all strains displayed high survival rates in the culture medium, their culturability reaching more the 9 $\log_{10}$ CFU/mL after two days. After 11 days, this value ranged from 7.8 to 8.2 $\log_{10}$ CFU/mL depending on the strain, and at least 50% of the *P. freudenreichii* population displayed an intact envelope. Since lysis of part of a bacterial population may be a microbial strategy to recover nutrients, in CIRM-BIA 138 (the strain with the highest population at day 11), cell lysis was assessed by quantifying intact bacterial cells using qPCR targeting the housekeeping gene *tuf*. No lysis was observed.

Conclusion: Taken together, our results suggest that *P. freudenreichii* strains use a viable but nonculturable (VBNC) state to adapt to the long term survival phase.

Significance and Impact of the study: Assessing the viability of *P. freudenreichii* and understanding their mechanisms for survival should be of great interest regarding their potential probiotic applications.

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Keywords: adaptation; bacteria; growth phases; culturability; long-term survival; viable but nonculturable; *Propionibacterium freudenreichii*.

Introduction

Propionibacterium freudenreichii is an Actinobacterium that is responsible for the opening and aroma of Swiss-type cheeses. PF is also used as a source of probiotics because PF has bifidogenic properties (Isawa *et al.*, 2002), PF is resistant to digestive stress (Jan *et al.*, 2000), and some strains are endowed with anti-inflammatory capabilities that could be used to prevent inflammatory bowel diseases (Okada *et al.*, 2013; Le Maréchal *et al.*, 2015). Its metabolic activity is characterized by its ability to ferment various carbohydrates, the main product being propionic acid (Houwen *et al.*, 1991; Loux *et al.*, 2015). PF is reportedly a hardy bacterial species, which can stay alive and metabolically active for long periods under stressful conditions (i.e. cave-aged Swiss-type cheeses at cool temperatures) (Dalmasso *et al.*, 2012). The genome sequence of the type-strain CIRM-BIA1 revealed the genetic basis for its hardiness (Falentin *et al.,* 2010a). In the laboratory, PF can be stored in a spent medium at room temperature for more than 6 months and remains culturable if inoculated in a fresh medium. Actinobacteria are known to be hardy and extremely resistant to nutrient shortage (Greenblatt *et al.*, 2004). However, except for the *Mycobacterium genus* which has been extensively explored (Ghodbane 2014), little is known about the mechanisms underlying the long term survival of this class.

During their growth and survival, Gram-positive and Gram-negative bacteria pass through different physiological phases (Kolter *et al.*, 1993; Hicks, 2005; Finkel, 2006; Bruno & Freitag, 2011; Wen *et al.*, 2013). In some bacterial species, conventional

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exponential and stationary phases are followed successively by the death phase and a long-term survival phase (Finkel, 2006; Helmus *et al.*, 2012). Long-term survival (LTS) can be defined as a phase during which the bacteria develop mechanisms to adapt and remain viable, even after a long period of nutritional shortage (Finkel, 2006; Meena & Rajni, 2010; Bharati *et al.*, 2012; Justice *et al.*, 2014): (i) sporulation (Fimlaid and Shen, 2015), (ii) quiescence/dormancy or (iii) programmed cell death (Finkel, 2006; Rittershaus *et al.*, 2013). During sporulation, a vegetative bacterial cell differentiates into a metabolically dormant cell-type known as a spore, which can reawaken when favourable conditions return (Fimlaid and Shen, 2015). Quiescence is characterized by decelerated or arrested growth in a viable non-replicating state (Rittershaus *et al.*, 2013), which can evolve to a viable but nonculturable (VBNC) state (Ramamurthy *et al.*, 2014). In programmed cell death (bacterial apoptosis), the death and lysis of part of a bacterial population may be profitable to surviving cells, which can catabolize the detritus. Cell death and lysis can be observed by monitoring the fall in the viable count using standard plating assays associated with a leakage of intracellular content that can be assessed biochemically (lactate dehydrogenase quantification) (Wilkinson *et al.*, 1994) or using qPCR methods (Falentin *et al.*, 2010b).

PF is a non-sporulating species, but PF is as yet unknown whether it survives by adopting a strategy of quiescence/dormancy or lysis of part of the bacterial population. During this study, we investigated the LTS (OD, culturability, membrane permeability and lysis) of eight *P. freudenreichii* strains throughout an 11-day period of culture without the addition of any nutrients, in order to investigate inter-strain variability in long-term survival capacities and to better understand the strategies used to ensure survival.

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Materials and Methods

Bacterial strains

Eight *P. freudenreichii* strains (Table 1) were used during this work. The strains were supplied by the International Centre for Microbial Resources - Bacteria of Food Interest – (Centre International de Ressources Microbiennes - Bactéries d'Intérêt Alimentaire; INRA Rennes).

Conditions for bacterial growth

All the strains were routinely cultured in YEL medium (pH=7.0) (Malik *et al.*, 1968) with no agitation and at 30°C. Each strain was grown in a different test tube for each manipulation, thus preventing the interference of oxygen in the environment and maintaining the *P. freudenreichii* strains under microaerophilic conditions. At T0, an inoculum of 7 $\log_{10}$ CFU/mL was added to all the tubes.

Growth kinetics were followed by Optical Density measurements using a Model DU 640 spectrophotometer (Beckman Coulter, Fullerton, California, USA) at 650 nm (OD_{650}) and by counting the number of CFU (CFU/mL) using the micromethod as described previously (Baron *et al.*, 2006). For enumerations, all the *P. freudenreichii* strains were plated in YEL agar culture medium at 30°C, under microaerophilic conditions, until the visualization of colonies (6 days). In all cases, two technical replicates and two biological replicates were performed.

Epifluorescence microscopy

The eight strains were monitored for 11 days using the LIVE/DEAD® BacLight™ Bacterial Viability Kit – Molecular Probes® (Life Technologies, USA), as recommended by the supplier. Microscopic observations were performed at each of the

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time points used for the OD_{650} and CFU/mL measurements. Briefly, SYTO9 (which stains all cells green, regardless of their membrane integrity) and propidium iodide (which stains permeabilized cells red) were mixed at a ratio of 1: 1 (5µL SYTO 9 and 5 µL propidium iodide). An 0.5µL aliquot of the mix was added to 100µL of the bacterial culture, vortexed, and incubated in the dark for 15 minutes. The bacteria were then analysed using epifluorescence microscopy with a phase contrast Nikon G11 microscope (Nikon D300) and Camera Control Pro 2 software (Nikon, Japan). For each sample, six photos (three photos with a x40 objective and three with a x100 objective) were analysed so as to visually estimate the percentage of permeabilized cells (red / red + green cells) at each time point.

DNA extraction of non-lysed cells

The DNA extraction method only enables the recovery of DNA from non-lysed cells. Samples were collected at three time points (79h, 144h and 168h post-inoculation) during the growth of *P. freudenreichii* CIRM-BIA 138, as described previously (Parayre *et al.*, 2007). Briefly, cells were harvested from 1 mL of the culture sample by centrifugation (5000g, 10 min.). The cell pellets were re-suspended in lysis buffer (Qiagen, Courtaboeuf, France) supplemented with lysozyme (Promega, France; 8mg/sample) and mutanolysin (Sigma, France; 20U/sample). These samples were incubated for 30 min. at 37°C for 1 h, before being treated with proteinase K (0.5mg/sample) prior to purification using the DNeasy Tissue Kit, as recommended by the supplier (Qiagen, Courtaboeuf, France). Three biological replicates and two technical replicates were used for each time point.

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Quantification of bacterial cells using quantitative PCR

To determine any cell lysis of CIRM-BIA 138 after entry into stationary phase and just before entering the LTS phase, quantitative PCR (qPCR) was used to quantify the non-lysed bacterial cells throughout growth, according to the method described by Falentin et al. (2010b). Briefly, the qPCR targeted *tuf,* a gene present in a single copy in the *P. freudenreichii* chromosome, using primers designed on the *tuf* sequence (F: CGAACGAGTTCCACTGCGGGT; R: GCAACATCGGCACCATCGGAC). Amplifications were performed exactly as described previously. Cycle thresholds (Ct) were determined after manual adjustment of the threshold within the linear part of qPCR logarithmic amplification curves. CIRM-BIA 138 samples were collected at 79, 144, and 168 hours for qPCR determination. Each experiment was carried out in triplicate (independent biological replicates). We checked by a spiking experiment that lysed cells could not contaminate qPCR results. A sample of entire cells (8 $\log_{10}$ CFU) was spiked with (9 $\log_{10}$ CFU) lyzed cells (the lysis was perfomed as described below). Extraction and qPCR on entire cells and spiked sample gave the same Ct. The qPCR results will give a quantification of entire cells only.

Results

Growth and survival rate of *Propionibacterium freudenreichii* under laboratory conditions.

The growth of the eight *P. freudenreichii* strains was monitored spectrophotometrically by measuring the optical density at 650 nm (OD_{650}), and by plate counting (CFU/mL) throughout an 11 day period. Strains were inoculated at 7 $\log_{10}$ CFU/mL in fresh YEL medium. Both of the monitoring methods showed that *P.*

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freudenreichii growth curves had roughly similar profiles despite variations in terms of their final culturability at 11 days. The curves established over a prolonged incubation revealed several phases, as shown for CIRM-BIA129 and CIRM-BIA138 (Figure 1. See all growth curves in the supplemental data, Figure S1). Under the conditions applied, the cultures entered the exponential growth phase to reach a maximum OD_{650} of 3 to 4 (for CIRM-BIA 138) and a population of 9 to 9.6 $\log_{10}$ CFU/mL between 50 h and 72 h. The OD_{650} curves then decreased steadily for about 100h, which corresponded to entry into the stationary phase, and reached a plateau of culturability at around 168 h, for a population of 8 to 8.7 $\log_{10}$ CFU/mL, depending on the strain (Figure 1) but always with a lower OD_{650}. The latter phase was designated the long term survival (LTS) phase. The other six *P. freudenreichii* growth curves were similar (Supplementary data Figure S1). In terms of culturability, all the strains survived well with a population of around 8 $\log_{10}$ CFU/mL after 11 days in the spent medium, i.e. without the addition of any nutrients (Figure 2, Table S1). Interestingly, two strains, CIRM-BIA121 and CIRM-BIA138, survived better than the six others (Figure 2) and maintained an average population of 8.2 $\log_{10}$ CFU/ mL at day 11, suggesting a strain-dependency in ability of *P. freudenreichii* for long-term survival. On Petri plates, colonies of *P. freudenreichii* maintained the same size and aspect whereas photon microscopy images revealed bacteria of the same size (not shown) but single cells in the exponential phase and groups of two to five cells in the stationary phase.

Evaluation of cell envelope permeability

LIVE/DEAD staining assays showed that the numbers of intact cells (stained green by SYTO9) remained stable (100% index) for each of the eight strains throughout the exponential and early stationary phases (Table S2). The numbers of intact cells then started decreasing at 50 or 150h post-inoculation and dropped to 80% and 58.6% for

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CIRM-BIA 129 and CIRM-BIA 138, respectively, 11 days post-inoculation. The numbers of cells with a non-permeabilized membrane (stained in green) decreased from 50 h to 150 h onwards, depending on the strains, to reach a minimum proportion of 50% at day 11 (Figure 3), suggesting that the number of dying or dead (but non-lysed) cells increased during the stationary and LTS phases.

Assessment of lysis by quantifying bacterial cells using qPCR

Because both plate counting assays and epifluorescence microscopy showed a reduction in culturability and an increase in membrane permeabilization, quantitative PCR was used to assess bacterial lysis. CIRM-BIA 138 (the strain with the highest population at day 11) cells were pelleted at three different time points before and after the observed loss of culturability. Quantitative PCR targeting the *tuf* gene was carried out on the DNA extracted from the pelleted cells. Under these conditions, lysed cells cannot normally be recovered (Falentin *et al.*, 2012). The copy number of the *tuf* gene did not vary significantly in the CIRM-BIA138 culture, demonstrating that the bacterial cells did not undergo lysis when entering the LTS phase (Figure 4).

Discussion

***P. freudenreichii* has a high survival rate at day 11**

P. freudenreichii belongs to the Actinobacteria, a group of Gram-positive bacteria with high GC content genomes. This group is known for its considerable versatility (Gao & Gupta, 2012), and includes members that can survive under extreme conditions (Greenblatt *et al.*, 2004). *P. freudenreichii* is reported to be a hardy species widely used

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in the cheese-making industry and is also well-documented for its probiotic potential (Jan *et al.*, 2000). Genome sequence analysis has revealed the genetic basis for its hardiness: (i) its ability to store polyphosphates as an energy reserve and to utilize them instead of ATP, (ii) its ability to store glycogen as a carbon source, and (iii) several copies of chaperones to cope with different stresses (Falentin *et al.* 2010a). The eight *P. freudenreichii* strains studied here survived well during a period of 11 days under nutritional shortage, according to the optical density and plate counting assays performed (around 8 $\log_{10}$ CFU/mL). In YEL medium, lactate - the preferential carbon source for PF- was exhausted two days after inoculation (Dalmasso *et al.* 2012). LIVE/DEAD staining followed by epifluorescence microscopy ascertained that more than 50% of the *P. freudenreichii* populations had an intact (non-permeabilized) membrane at this date. The growth curves for CIRM-BIA 129 and 138 (Fig. 1) displayed some fluctuations during long-term incubation. We suggest that intermittent cryptic regrowth might be responsible for this variability. These cryptic growths may result from some specific metabolisms induced to adapt to starvation or the growth of a sub-population of mutants derived from the inoculated strain and with a growth advantage that was explained in the review by Finkel *et al.* (2006).

The best surviving strain did not use partial lysis to sustain viability

Previous studies had shown that some lactic acid bacteria present in dairy products can lyse under difficult conditions and that the cell debris released into the culture medium may serve as a nutrient source to ensure survival of the non-lysed sub-population (Lortal *et al.*, 1997; Deutsch *et al.*, 2002; Lortal & Chapot-Chartier, 2005). A study of the autolysis of *Lactobacillus* and dairy propionic bacteria showed that this

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autolysis is associated with several factors, including among others the type of strain, autolysins, pH, temperature and ionic strength (Lortal *et al.*, 1997). For CIRM-BIA 138, the quantification of intact cells using quantitative PCR showed that under the study conditions applied here, lysis could be ruled out. Whether this is a general feature of *P. freudenreichii* still needs to be determined. The absence of lysis (i) could be confirmed by quantitative PCR on cells exposed to propidium monoazide, so as to prevent any contamination by DNA from dead cells, (ii) needs to be confirmed in other *P. freudenreichii* strains and under different conditions before it is generally accepted.

P. freudenreichii probably enters a viable but nonculturable (VBNC) state

The experimental data reported here offer a range of presumptions suggesting that *P. freudenreichii* enters into a VBNC state. VBNC is a survival strategy adopted by numerous bacteria in response to adverse conditions such as starvation, extreme temperature, pH, oxygen stress, antibiotic pressure, etc. Once in a VBNC state, the bacteria no longer grow in routine laboratory medium and conditions, but they nevertheless remain viable and can be resuscitated when more favourable conditions are provided (Ramamurthy *et al.*, 2014). The methods generally used to detect VBNC bacteria include bright field microscopy with nalidixic acid; fluorescent microscopy, and especially use of the BacLight LIVE/DEAD kit; and molecular techniques (DNA or RNA) (Oliver, 2009; Fakruddin *et al.*, 2013). All of the eight *P. freudenreichii* strains studied here displayed a loss of culturability of ~1 $\log_{10}$ CFU/mL (Table S1 and Figure 5), when this parameter was compared between 50-72 hours and 11 days post-inoculation. LIVE/DEAD staining showed that about half of the bacterial cells had a permeabilized membrane. Using quantitative PCR, an absence of lysis was shown on

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strain CIRM-BIA138. These findings suggest that at least a part of the *P. freudenreichii* population entered a VBNC state and that the entry into LTS is concomitant with entry into a VBNC state, at least for 1 $\log_{10}$ (i.e. 90%) CFU/mL. Since the total bacterial population was stained either green (50%) or red (50%), we suggest that VBNC cells (90%) can take up both colours and that LIVE/DEAD staining cannot be used to quantify VBNC cells.

The VBNC state has been described in various Gram positive bacteria, such as *Enterococcus faecalis*, *Enterococcus hirae*, *Listeria monocytogenes*, and *Mycobacterium tuberculosis* (Oliver et al., 2009), and in Gram negative bacteria such as *Vibrio cholerae* (Gonzales-Escalona et al. 2006), *Escherichia coli* (Darcan *et al.*, 2009) and *Helicobacter pylori* (Casasola-Rodríguez *et al.*, 2013). Many of the studies on the VBNC state have focused on pathogenic bacteria (Ramamurthy et al. 2014). This state has also recently been reported for bacteria of food interest and particularly in ripened cheese, suggesting a role for these bacteria in the organoleptic qualities of cheeses (Martin-Platero *et al*. 2008, Desfossés-Foucault *et al.* 2012, Ruggirello *et al.* 2014).

In conclusion, our findings suggest that one strategy for bacterial survival is dormancy, rather than partial bacterial lysis, in order to sustain the remaining cells. During the past decade, many studies have investigated the bacterial mechanisms underlying LTS in various fields: environmental, industrial, medical and biotechnological microbiology. This study represents the first work involving the phenotypic characterization of *P. freudenreichii* over an 11-day period without the addition of nutrients, and revealed different phases of growth, membrane permeabilization and entry into dormancy and a VBNC state to ensure LTS. A VBNC state may however have an important impact on the technological uses of these bacteria, as either starters in cheese-making or probiotics, since viability and metabolic activity

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are important traits in their wide range of potential uses. This therefore opens the way for further investigations on a molecular basis of the LTS of *P. freudenreichii*. The availability of genome sequences (Loux *et al.*, 2015) will greatly assist such investigations using comparative genomics and transcriptomics approaches in order to understand the organization, genes and metabolic pathways required for LTS. Assessing viability and understanding the mechanisms underlying the survival of *P. freudenreichii* should be of considerable interest regarding the development of effective bacterial probiotics.

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Conflict of Interest section

The authors have no conflict of interests to declare.

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Figure legends

Figure 1: Growth curves of *P. freudenreichii* strains CIRM-BIA129 and CIRM-BIA138 determined at OD650 (upper panels), and plate counting (CFU/mL, lower panels).

Figure 2: Viability of the eight *P. freudenreichii* strains (in CFU/mL) after 11 days in the spent culture medium (YEL) based on a mean of two technical replicates and two biological replicates. Upper bars are standard deviations.

Figure 3: (A) Proportion of non-permeabilized cells during the growth and survival of *P. freudenreichii* strains CIRM-BIA129 **(A)** and 138 **(B)** measured by LIVE/DEAD assay followed by epifluroscence microscopy (mean values of three epifluorescence microscopy fields at x40 magnification, and three fields at x100 magnification). Representative epifluorescence microscopy views of *P. freudenreichii* (CIRM-BIA 138) cultures after LIVE/DEAD staining during exponential growth **(C)** and after 11 days of growth **(D)**.

Figure 4: Quantification of bacterial cells during the growth of *P. freudenreichii* CIRM-BIA138. Each point gives the copy number of *tuf* gene determined by qPCR on DNA extracted from *P. freudenreichii* CIRM-BIA138 (three biological replicates).

Figure 5: Fate of CIRM-BIA138 bacterial cells between 50-72 h post-inoculation and the LTS phase 11 days post-inoculation assessed by (A) culturability on Petri dishes, (B) LIVE/DEAD staining followed by epifluorescence and (C) quantification of intact

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cells by quantitative PCR.

Table 1. *Propionibacterium freudenreichii* strains used during this work.

Strains of *P. freudenreichii*	**Source**
CIRM-BIA 1	Reference strain
CIRM-BIA 9	Emmental cheese 1989 Van Niel
CIRM-BIA 119	Gruyère cheese 1973
CIRM-BIA 121	Swiss cheese 1937
CIRM-BIA 127	Emmental cheese 1992
CIRM -BIA 129 alias ITG-P20 *	Emmental cheese 1992NA
CIRM-BIA 138 alias ITG-P9 *	Emmental cheese 1992NA
CIRM-BIA 139 alias ITG-P23 *	Emmental cheese 1992NA

*bacterial strains obtained from the Centre International de Ressources Microbiennes (CIRM) dedicated to Bacteria of Food Interest (Bactéries d'Intérêt Alimentaire, BIA) - (CIRM-BIA, UMR1253, INRA Rennes, France). * Strain CIRM-BIA (alias ITG-P20 129), strain CIRM-BIA 138 (alias ITG-P9) and strain CIRM-BIA 139 (alias ITG-P23) were kindly supplied by Actalia (Rennes, France).

Supporting Information

Additional supporting information can be found in the online version of this article.

Fig. S1. Kinetics of growth and survival measured by optical density in eight strains of *P. freudenreichii*

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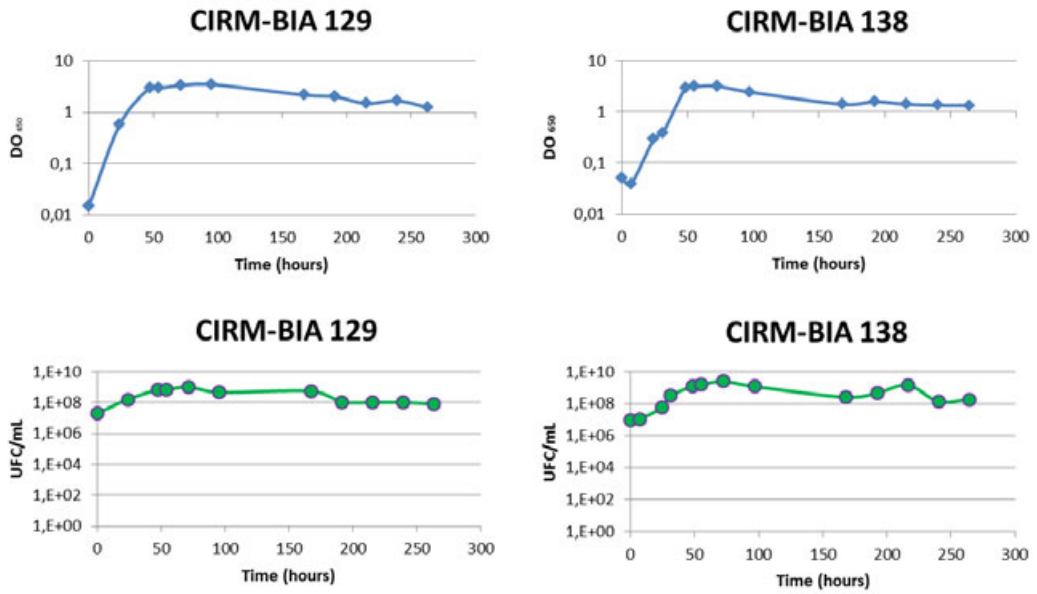
Table S1. Kinetics of the growth and survival of eight *P. freudenreichii* strains measured by culturability in Petri dishes.

Table S2. Proportion of non-permeabilized cells during the growth and survival of eight *P. freudenreichii* strains measured by LIVE/DEAD staining followed by epifluroscence microscopy.



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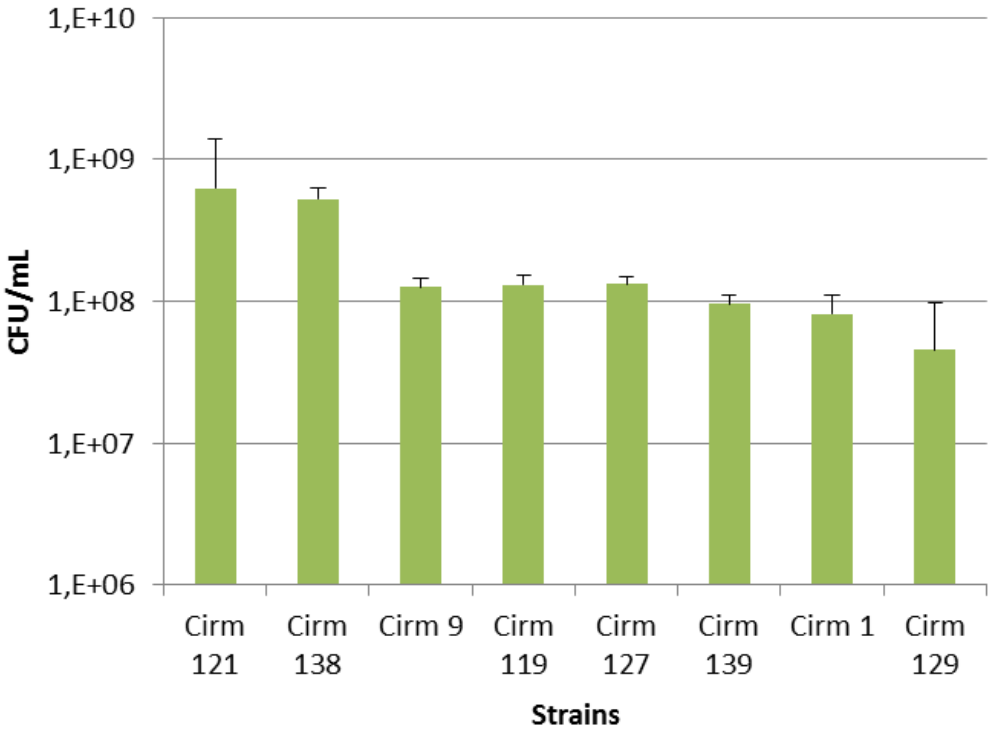

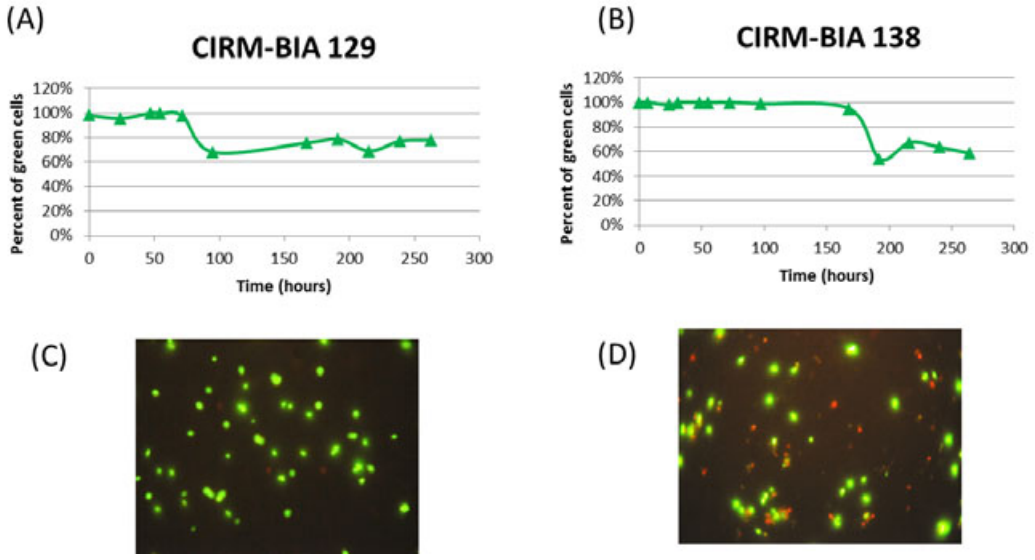

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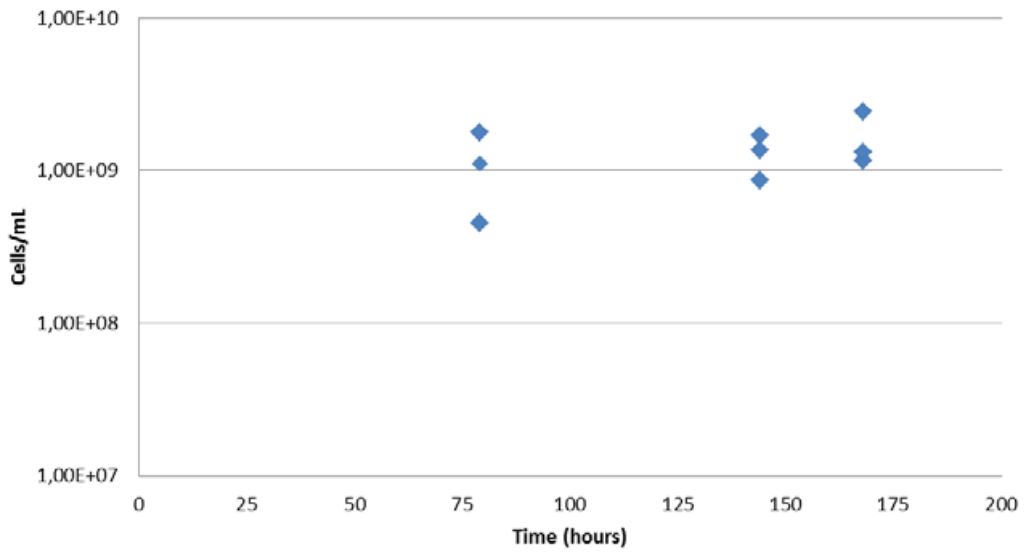

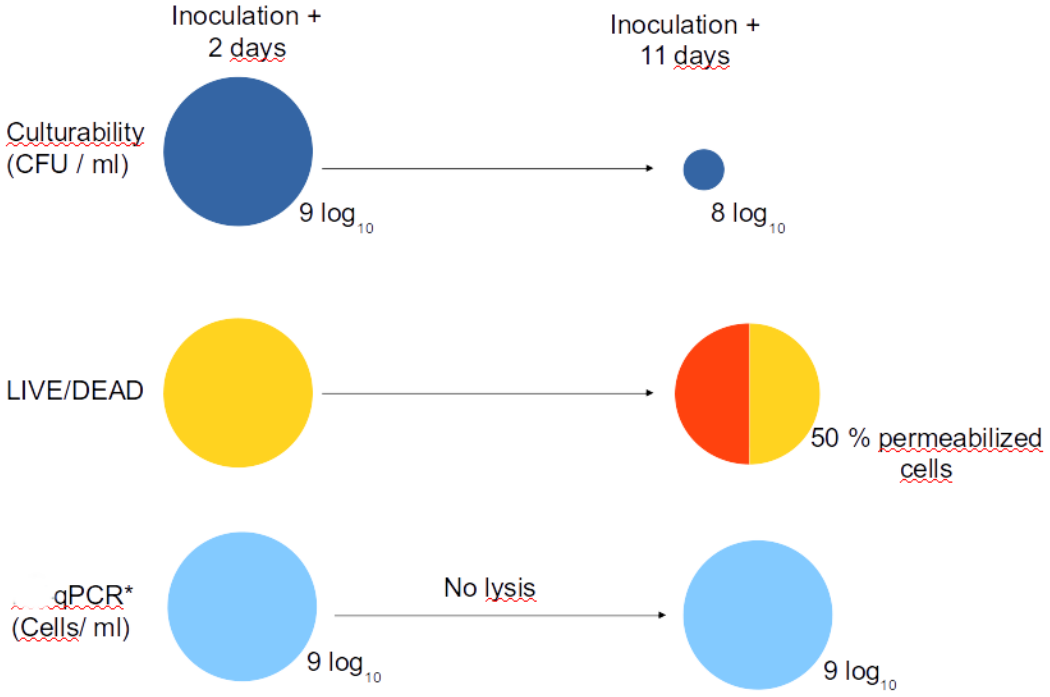

Figure 5

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