



Contents lists available at ScienceDirect

Cryobiology

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

Brief Communication

Cryopreservation of anoxygenic phototrophic Fe(II)-oxidizing bacteria[☆]F. Hegler, A. Kappler^{*}

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ARTICLE INFO

Article history:

Received 16 December 2009

Accepted 21 April 2010

Available online 28 April 2010

Keywords:

Phototrophic Fe(II)-oxidizing bacteria

Storage

Cryopreservation

Glycerol

Freezing

ABSTRACT

Preservation and storage of microbial stock cultures is desirable since the risk of contamination or loss of living cultures is immanent while over long periods mutations accumulate. Generally, it is rather difficult to preserve photosynthetic bacteria due to their sensitive photosynthetic membranes [1]. Phototrophic Fe(II)-oxidizing bacteria face an additional challenge; since they are exposed to light and Fe(II) during growth, they have to cope with radicals from Fenton reactions of Fe(II)-species, light and water. Therefore, phototrophic Fe(II)-oxidizing strains are thought to be especially susceptible to genetic modifications. Here, we provide a simple and fast protocol using glycerol as cryo-protectant to cryopreserve three strains of anoxygenic phototrophic Fe(II)-oxidizing bacteria from different taxa: α -proteobacteria, γ -proteobacteria and chloroflexi. All three strains investigated could be revived after 17 months at -72 °C. This suggests that a long-term storage of phototrophic Fe(II)-oxidizing strains is possible.

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Iron is the fourth most abundant element in the earth crust and can be used by microorganisms either as electron donor or electron acceptor for energy generation and growth [2]. Phototrophic Fe(II)-oxidizing microorganisms were discovered about 15 years ago and since then have been found in various environments [see Ref. in 2]. Originally the strains were isolated from Fe(II)-rich surface-near freshwater environments. These organisms are especially prone to mutations due to impairments of the cells through hydroxyl radicals produced by Fenton reactions [3] according to the following equation:



Such radicals are known to cause stress and undesired mutations in the bacteria [4]. Therefore, in order to preserve the isolated strains with their original genetic systems, it is desirable to have a robust and easy to use method for long-term storage of phototrophic Fe(II)-oxidizing bacteria minimizing the risk of mutations.

Generally, all methods for storage of bacterial strains have advantages as well as disadvantages. When living subcultures are kept as stock, they have to be transferred to fresh growth medium in regular time intervals. Contamination and accumulation of mutations that could potentially lead to a loss of function are a constant risk. In contrast, cryopreservation of stock culture makes

regular transfer of stock cultures unnecessary and therefore provides a solution to avoid potential risks when transferring stock cultures [5]. When freezing and thawing the cultures, ice-crystal formation has to be minimized as it can destroy cell and photosynthetic membranes. Often, chemicals such as glycerol are used to protect these membranes during freezing. In the case of phototrophic Fe(II)-oxidizing bacteria, not only the protection of the membranes during freezing is of paramount importance but in addition anoxic conditions must be maintained due to oxygen sensitivity of some of the cells. This is potentially necessary to ensure that the cells could be revived using Fe(II) as electron donor for their anaerobic metabolism.

The following cryopreservation methods have been applied in previous studies for long-term storage of bacteria: (I) freezing in liquid N_2 [1], (II) freezing at -72 °C [6], (III) freezing the cultures on glass-beads at -72 °C [7], or (IV) lyophilisation [8]. Each preservation method has its own requirements with respect to technical equipment and culture preparation.

The goal of this study was to develop a method that makes preservation of phototrophic Fe(II)-oxidizing bacteria easily accessible without sophisticated equipment and thus be generally applicable. Therefore permanent storage in liquid nitrogen was not considered a method of choice because such storage tanks are not ubiquitously available to many microbiology laboratories. The fact that cultures of anoxygenic phototrophic Fe(II)-oxidizing bacteria preferably are kept anoxic further limits the choice of available methods for cryopreservation. Therefore lyophilisation and storage on glass-beads were also excluded because these techniques cannot be applied under anoxic conditions without significant methodological and/or technical efforts. Given these limitations, freezing at -72 °C seemed to be the most promising option with the lowest

[☆] Statement of funding: The study was funded by the graduate program ("Promotionsverbund") of the University of Tuebingen "Cell-Material-Interactions". No other funding agencies were involved in supporting this study.

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requirements for long-term storage of anoxygenic phototrophic Fe(II)-oxidizing bacteria. Various concentrations of glycerol as a cryo-protectant were tested based on a previous study by Feltham et al. [6]. Other cryo-protectants were not tested since glycerol showed promising results in initial experiments.

In order to test whether phototrophic Fe(II)-oxidizing bacteria from different taxa can be frozen at -72°C , we grew cultures of the α -proteobacterium *Rhodobacter* sp. strain SW2, the γ -proteobacterium *Thiodictyon* sp. strain F4 and *Chlorobium ferrooxidans* strain KoFox of the chloroflexi in 22 mM bicarbonate buffered mineral medium according to [9] with 10 mM Fe(II) as electron donor. Cell counts and Fe(II) quantification over time ([9] were used for determination of the growth phase.

Glycerol (87% p.a. quality, Fluka, Steinheim, Germany) was sterile filtered (0.22 μm , cellulose ester, Fisher Brand, Germany) and deoxygenated by alternating cycles of vacuum and flushing the headspace of the bottle with N_2 for 3×20 min and 3×2 min, respectively. The anoxic glycerol was added to a sterile and anoxic 6 mL N_2/CO_2 -flushed glass vial (BGB-Analytik, Boeckten, Switzerland) closed with a butyl-rubber-stopper.

Samples from the cultures were taken at the late exponential or early stationary growth phase when all Fe(II) was visibly oxidized and transferred anoxically with a N_2/CO_2 -flushed (90:10) sterile syringe into the anoxic glass vial containing glycerol. After complete oxidation of the Fe(II), a culture of *Rhodobacter* sp. strain SW2 contained $1.9\text{E} + 8 \pm 1.4\text{E} + 7$ cells mL^{-1} , *C. ferrooxidans* sp. KoFox cultures contained $1.1\text{E} + 8 \pm 1.9\text{E} + 7$ cells mL^{-1} and *Thiodictyon* sp. strain F4 cultures contained $1.5\text{E} + 7 \pm 1.3\text{E} + 6$ cells mL^{-1} . The final volume of glycerol–cell-culture suspension was 1 mL containing between 10% and 20% (v/v) glycerol. The concentrations of glycerol were chosen according to [6]. After 20 s of careful shaking, the vial was shock-frozen in liquid nitrogen and immediately transferred to a -72°C freezer. Our results showed that *Rhodobacter* sp. strain SW2 and *C. ferrooxidans* sp. KoFox could be revived

well using 20% glycerol while *Thiodictyon* sp. strain F4 required only 10% glycerol.

In order to revive the strains, we allowed the vials to slowly thaw at room temperature. The cell survival rate of a freeze/thaw cycle was determined by counting dead and live cells after staining cells according to the LIVE/DEAD[®] BacLight[™] Bacterial Viability and Counting Kit (Molecular Probes: (L34856)). The number of dead cells in a stationary phase culture and in an autoclaved control sample was compared to the cell numbers of a culture that underwent freezing and thawing in an anoxic glycerol-containing vial.

We also determined the extent of Fe(II) oxidation after 17 months of storage at -72°C . In order to do so, we anoxically transferred the total volume of the thawed culture–glycerol-mixture (1 mL) to a 60 mL serum bottle containing 25 mL medium amended with 10 mM Fe(II) buffered with 22 mM bicarbonate [9]. The liquid culture first established after thawing was again transferred to fresh growth medium after about 3 weeks (1% v/v inoculum). This procedure reduced the glycerol concentration from initially 20% during freezing to 0.004% (v/v) in the second culture after thawing. Glycerol is a potential substrate for the Fe(II)-oxidizing cells that could be used during photoorganotrophic growth. More importantly, however, organic compounds such as glycerol are potential reductants for the Fe(III) minerals formed by microbial Fe(II) oxidation. During this process glycerol is degraded and toxic intermediates are formed [10]. Therefore, by transferring the revived culture a second time before determining the extent of Fe(II) oxidation, potential artifacts could be prevented: either microbial glycerol oxidation (or oxidation of its products resulting from the abiotic reduction of Fe(III)) or more importantly, the alteration of the amount of Fe(II) oxidation in the medium due to the abiotic reduction of Fe(III) coupled to the oxidation of glycerol.

In the second transfer after thawing, we quantified the amount of Fe(II) oxidizable by the thawed, revived cells. Using a spectrophotometric assay with ferrozine, we determined the Fe(II) concentration directly after inoculation and when Fe(II) oxidation halted [9]. We found that all cultures oxidized Fe(II) phototrophically to Fe(III) oxy-hydroxides after the second transfer (Fig. 1) to an extent of >99% within 21 days, demonstrating the successful revival of the cultures after freezing. Estimation of oxidation rates of >0.5 mM/day based on the concentrations of Fe(II) used (10 mM) and the time after which complete oxidation was observed (in maximum 21 days) we conclude that the oxidation rates of all cultures after freezing are comparable to the oxidation rates before freezing where maximum Fe(II) oxidation rates of 0.5–1.0 mM Fe(II) per day for strain SW2, 1.0–1.5 mM per day for KoFox and 2.0–2.5 mM per day for strain F4 were described [9].

Cell viability analysis in experiments with *Rhodobacter* sp. strain SW2 cells harvested at late exponential stage using the dead-live staining kit showed that freezing killed about 5–10% of the cells (Fig. 2). For cells harvested at early stationary growth phase similar survival rates were observed. *C. ferrooxidans* sp. KoFox and *Thiodictyon* sp. strain F4 survival rates could not be determined using the

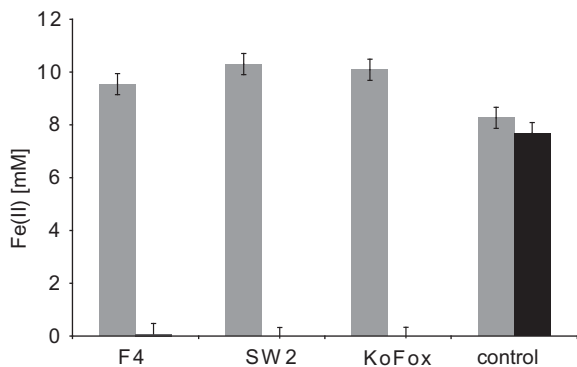


Fig. 1. Fe(II) oxidation by three phototrophic Fe(II)-oxidizing strains *Thiodictyon* sp. strain F4, *Rhodobacter* sp. strain SW2 and *C. ferrooxidans* sp. KoFox after freezing and thawing. The graph shows the Fe(II) concentration at the beginning (0 h, grey bars) and after complete Fe(II) oxidation (21 days, black bars) ($n = 2$). The control shows the Fe(II) concentrations in a non-inoculated culture bottle.

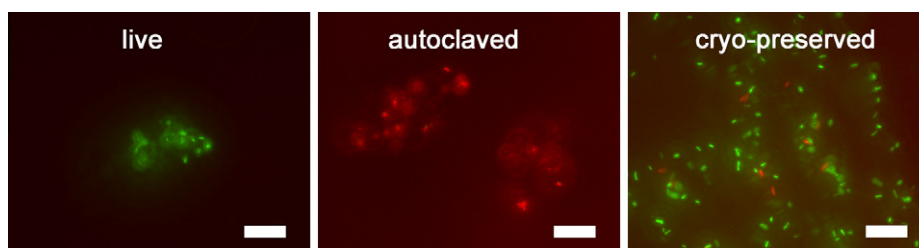


Fig. 2. Dead-live staining of *Rhodobacter* sp. strain SW2. Live cells were stained from a growing culture at late exponential stage (left), as control cells were autoclaved (middle), and cryo-preserved cells were analyzed directly after thawing (right). Scale-bar = 10 μm .

dead-live stain because the staining kit did not allow a clear distinction between dead and living cells, probably due to the membrane properties of the strains KoFox and F4. Since these two cultures represent mixed cultures and consist of more than one strain, a dilution series to determine the viable number is not possible without ambiguity since the role of the individual strains in the Fe(II) oxidation process is unknown.

Nonetheless, the results presented in this study clearly demonstrate that the cryopreservation method developed for phototrophic Fe(II)-oxidizing bacteria is easy to perform, robust and suitable for storage of these bacteria for at least 17 months, potentially even longer, offering a simple and inexpensive method to laboratories not specialized in permanent cryopreservation of microorganisms in liquid nitrogen.

Acknowledgments

We would like to thank Dr. Sebastian Behrens and Emily-Denise Melton for their help in improving the quality of the paper. This work was supported by the Graduate Program ("Promotionsverband") Cell-Material-Interactions funded by the University of Tuebingen.

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