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Short Communication

# Expression of stress-related proteins in *Sediminibacterium* sp. growing under planktonic conditions

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Aggregation is a common trait of bacteria in natural and engineered biological systems. Microbial aggregates, such as flocs, granules, and biofilms, are spatially heterogeneous environments. It is generally observed that by growing under aggregated conditions bacteria respond and adapt to environmental stress better than free-swimming bacteria of the same species. We performed a proteomic analysis of a strain of *Sediminibacterium*, isolated from activated sludge, which grew planktonically in diluted culture media and in an aggregated form in media containing a high concentration of organic substrate. Auto-aggregation was also observed in the presence of pyruvate in dilute media. Expression of a number of stress-related proteins significantly increased under planktonic growth in comparison to aggregate growth. The upregulated proteins, identified by MALDI-TOF mass spectrometry, were two isoforms of a protein belonging to the universal stress family (UspA), a thioredoxin-disulfide reductase, the *Campylobacter jejuni* orthologue transcriptional regulator (Cj1172c), and the CocE/NonD hydrolase. We conclude that *Sediminibaterium* sp. C3 growth is stressed under planktonic conditions and that aggregation induced by pyruvate protects the bacteria against oxidative stress.

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## Introduction

In natural environments, bacteria exist predominantly in aggregates and biofilms. Microbial auto-aggregation and co-aggregation has found widespread use in many industrial applications, including wastewater treatment, because the formation of multicellular associations of micro-organisms facilitates rapid settling and efficient separation of cells from process liquor. Biofilms, granules and activated sludge flocs are complex structures of self-immobilized microbial communities within a spatially heterogeneous environment. As a consequence of environmental gradients, immobilization exposes bacteria to growing conditions that differ from conditions in the bulk water phase [1, 2]. How bacteria respond and adapt to varying nutrient and oxygen concentrations within flocs and other aggregates is not well understood [3]. Microaerophilic growth appears to be a common trait of phylogenetically diverse bacteria in a variety of low-substrate environments, including activated sludge [4, 5]. Exposure to high oxygen concentration at low substrate concentration elicits an increase in the concentration of the stress indicator cyclopropane fatty acid in the model bacteria *Pseudomonas jessenii* [5]. Initially proposed as a mechanism called low-substrate regulated microaerophilic behavior as a stress response [5], this strategy would allow the survival of bacterial cells in oligotrophic environments at low oxygen levels by slowing the rate of oxidative metabolism, and hence generating less reactive oxygen species (ROS). This mechanism differs from the classic model in that the microaerophilic growth is only manifested at low substrate concentrations. However,

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while the initial hypothesis was that the main intracellular ROS formed within the aerobic respiratory chain, experiments performed in *Escherichia coli* mutants lacking respiratory enzymes indicated that ROS are primarily produced by accidental autoxidation of non-respiratory flavoproteins (reviewed in [6]). The low-substrate regulated microaerophilic behavior has been thereafter challenged by the suggestion that the presence in the cultivation media of substrates, derived from proteolytic digestion, such as peptone or yeast extract, scavenge oxygen reactive species [4, 7].

During the course of experiments designed to evaluate the effect of shock toxic loads and high temperature on the stability of activated sludge flocs, we isolated a bacterium of the genus *Sediminibacterium*. A high quality draft of the genome sequence of strain *Sediminibacterium* sp. C3 is now available [8]. This strain was unique, in that it exhibited microaerophilic planktonic growth in low concentration media, but aggregated growth occurred in media containing high concentration of organic substrates or in diluted media containing relatively low concentration of pyruvate (0.07% w/v).

Apart from being a central carbon metabolite, pyruvate is a very efficient scavenger of hydrogen peroxide [9–11]. This fact would therefore be consistent with the hypothesis that aggregated growth of *Sediminibacterium* sp. C3 in media containing pyruvate, as well as high substrate media, is linked to the increased tolerance to oxygen reactive species. With the aim of gaining a better understanding of microaerophilic growth that is limited to dilute media, we examined the expression profiles of planktonic and aggregated cells. Based on the differences in the expression of a number of stress-related proteins between planktonic and aggregated growth, we conclude that *Sediminibacterium* sp. C3 growth is stressed under planktonic conditions and that aggregation induced by pyruvate protects the bacteria against oxidative stress.

## Materials and methods

### Strain of *Sediminibacterium* sp. C3 and growth conditions

The strain of *Sediminibacterium* used in this study was isolated from activated sludge collected from a wastewater treatment plant of a petroleum refinery, which was incubated in a laboratory-scale bioreactor for 9 days and the isolate identified using a high-throughput, plate wash PCR procedure [12]. Further details of the isolation and culture conditions of *Sediminibacterium* sp. C3 will be given elsewhere, and the strain is deposited in the National Bank of Microorganisms (WDCM938), Facultad de Agronomía, Universidad de Buenos Aires, under the collection number BNM541.

To obtain planktonic and aggregated growth, R2A medium [13] with increasing concentrations of pyruvate (0–0.08%) (w/v) were inoculated with an overnight culture and grown at 30 °C with agitation at 200 rpm for 2–4 days. Meat peptone medium (4 g $L^{-1}$) supplemented with vitamins and Wolfe's mineral solution [13] (referred hereafter as meat peptone medium) was used for most assays, unless otherwise noted.

### Catalase activity and sensitivity to hydrogen peroxide

Catalase activity was tested on colonies grown in meat peptone medium by adding a drop of $H_2O_2$ 30% v/v [4]. Formation of bubbles indicated a positive test. To determine the sensitivity to $H_2O_2$, 0.1 ml of an overnight culture of *Sediminibacterium* was spread on R2A plates and sterile filter paper discs containing a freshly prepared solution of 3% $H_2O_2$ (v/v) were placed in triplicate on the plates. The plates were incubated at 30 °C for 4 days and the sensitivity was determined as zones of inhibition around discs [4].

### Growth assay

Cultures of *Sediminibacterium* sp. C3 were incubated at 30 °C in meat peptone medium plus the indicated concentration of sodium pyruvate (0–0.07% w/v) for 16 h. The flocs were allowed to settle under gravity for 15 min, and planktonic growth was determined by optical density (OD) readings of the supernatant at 600 nm in an Ultrospec 2000 Spectrophotometer (GE Healthcare Life Sciences).

### Two-dimensional PAGE and mass spectrometry

*Sediminibacterium* sp. C3 pure cultures were grown in 50 ml of meat peptone medium. Sodium pyruvate, up to 0.07% (w/v) was added to culture medium in order to obtain aggregated growth, and was omitted for planktonic growth. Incubations were performed at 30 °C using an orbital shaker at 75 rpm. Total proteins were extracted using phenol under alkaline conditions. Bacterial pellets were resuspended in saline solution, mixed with lysis buffer (25 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.1% Triton X-100) and transferred to 2 ml screw cap tubes with 0.5 g zirconia silica beads. Cells were physically disrupted by 5 cycles of 30 s shaking in a Precellys homogenizer (Bertin Technologies) at 5000 rpm. The aqueous phase was transferred to a clean tube and mixed with phenol equilibrated with TE buffer at pH 8 and chloroform-isoamyl alcohol (24:1) and centrifuged at 5000*g* for 30 min at 4 °C. Phenolic phase was collected and precipitated

 

overnight with four volumes of 0.1 M ammonium acetate in methanol at −20 °C. The pellet was centrifuged, washed with cold acetone, resuspended on alkaline buffer (50 mM Tris pH 10), and reprecipitated. The pellet was resuspended on isolectric focusing buffer (100 mM Tris-HCl pH 8.0, 1mM EDTA, 7 M urea, 2 M thiourea, 10% glycerol (w/v), 4% CHAPS), and stored at −80 °C until analysis.

Immobiline DryStrip pH 4-7 (GE Healthcare Life Sciences), 7 cm long, were rehydrated for 16 h with 125 μL of protein solution containing 250 μg of total protein dissolved in isoelectric focusing buffer supplemented with 60 mM dithiotreitol (DTT), 0.5% ampholine pH 4-7, 0.01% bromophenol blue (BPB). Isoelectric focusing was carried out with an Ettan IPGphor 3 (GE Healthcare Life Sciences) at 20 °C, applying a voltage gradient up to 5000 V. The total product time × voltage applied was 8500 Vh for each strip. For the second dimension, the strips were equilibrated during 45 min with gentle shaking in buffer containing 1% DTT, 4.5% iodoacetamide, 6 M urea, 2% sodium dodecyl sulfate (SDS), 30% glycerol, 50 mM Tris-HCl, pH 8.8. The equilibrated strips were laid on a 10% polyacrylamide gel containing 0.5% agarose. Electrophoretic runs were performed for 1 h at 15 mA/gel, and then at 25 mA/gel. Gels were stained with Coomasie blue G-250 and scanned with Image Scanner III (GE Healthcare Life Sciences). Comparisons between gels (detection, quantification, and spot matching) were performed using Image Master 2D Platinum 7.0 software (GE Healthcare Life Sciences). Protein spots of interest were cut out, digested with trypsin and analyzed by MALDI-TOF-MS (Institut Pasteur de Montevideo, Uruguay), using the annotated draft genome [8] as an in-house database.

Four biological replicates were used for statistical analysis. Stained protein spots were quantified on the basis of their volumes, an integration of optical density and area. The relative volume of each spot was calculated as a percentage of the total volume of all detected spots to correct for differences in gel loading and staining. Changes in relative spot volumes with p-values $<0.05$ from one-way ANOVA were considered significant for differential expression.

### Quantification of universal stress protein transcript

For the reverse transcription quantitative PCR (RT-qPCR), cultures were transferred to Erlenmeyer flasks containing 50 ml of meat peptone medium. Bacteria were grown under two treatment conditions: with or without pyruvate [0.07% (w/v) final concentration]. Samples were collected at early exponential, late exponential and stationary phases. Specific primers for the universal stress protein gene USP gene of *Sediminibacterium* sp. C3 USP23F [8] (5′-CCGATTTTTCTGCTACCGCC-3′) and USP221R (5′-TCAGCCCTGAACTGCAAGAG-3′) were designed using the Primer-BLAST tool available from the NCBI web site (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the draft *Sediminibacterium* sp. C3 genome sequence.

RNA extraction and first strand cDNA synthesis was performed as in Ref. [14]. The reverse transcription was primed using USP221R. Real-time PCR followed the procedures described in Ref. [15]. The program consisted of 15 min at 94 °C, 30 s at 94 °C, and 35 cycles at 58 °C for 20 s, 72 °C for 30 s. A plasmid containing the target sequence of the USP gene (199 bp) was used as a standard. The annealing temperature and optimal concentrations of $Mg^{2+}$ used in the real-time PCR was determined from a temperature gradient PCR using genomic DNA of the *Sediminibacterium* isolate. No template and no enzyme controls were included in all real-time RT-PCR reactions. In order to normalize the expression of the USP gene, the number of copies of 16S rRNA was calculated using a *Sediminibacterium*-specific primer S-S-Sdm-0469-a-A-18.

## Results and discussion

### Screening and isolation of *Sediminibacterium* sp.

Different media and incubation conditions for *Sediminibacterium* were screened using a high-throughput procedure, using the newly designed primer S-S-Sdm-0469-a-A-18. Isolation of *Sediminbacterium* sp. C3 was successful in R2A when the medium was amended with an extract of activated sludge from the refinery WWTP, which was also the source of the inoculum. Further details of the isolation will be reported separately. Growth of the isolate in defined media, such as R2A or synthetic sewage [15] required the addition of vitamins and trace minerals based on Wolfe's solution.

*Sediminibacterium* sp. C3 colonies were circular, smooth, and orange-pigmented. In semisolid medium (synthetic sewage plus 0.3% agar) the isolate formed a band of growth 3 mm below the surface (Supporting Information Fig. S1), suggesting miroaerophilic growth. The isolate shared with microaerophilic bacteria the traits of being catalase negative and the high sensitivity to hydrogen peroxide.

In broth cultures, it had the ability to growth either a planktonic or aggregated mode, depending on the composition of the medium. Increasing the concentration of pyruvate in diluted meat peptone medium or increasing the concentration of peptone, reduced planktonic growth, and enhanced the aggregated growth (Fig. 1 and Supporting Information Fig. S2).

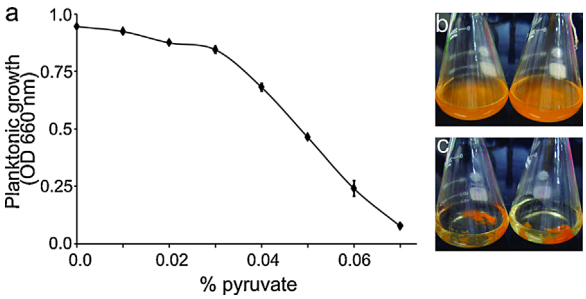


**Figure 1.** Planktonic growth of *Sedminibacterium* sp. in pure culture as a function of pyruvate concentration in the medium (a). Error bars are standard error of the mean of measurements from two separate experiments. Growth of *Sedminibacterium* sp. C3 in duplicate pure culture, with no pyruvate added to meat peptone medium (4 g/L) (b) and the same medium with 0.07% pyruvate (c).

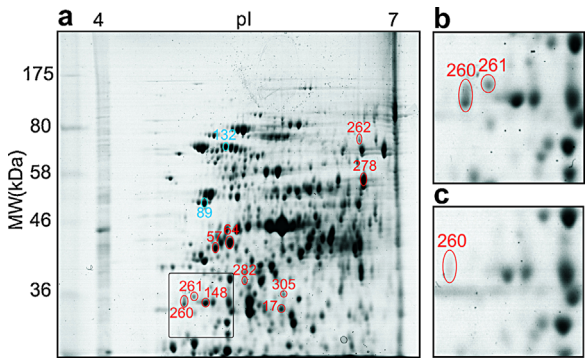


**Figure 2.** Proteomic profiling of *Sediminibacterium* sp. C3 grown in planktonic mode separated by 2D-PAGE (a). Proteins were separated by isoelectric focusing (pI range, 4–7) in the first dimension and by 10% SDS-PAGE in the second dimension. The migration positions of molecular mass markers and pI values are shown. Spot numbers indicate proteins identified following in-gel tryptic digestion and LC-MS/MS analysis (see Table 1). Spots corresponding to proteins overexpressed in the planktonic form are indicated in red. Spots corresponding to proteins overexpressed in the aggregated form are indicated in blue. Box represent 2D-PAGE region of spots corresponding to UspA proteins zoomed in a representative gel of planktonic (b) and aggregate growth (c).

The growth of isolates in R2A agar plates was restricted to the heavily inoculated area of plates and normal growth was restored supplementing R2A agar plates with pyruvate, 0.03% (w/v) (Supporting Information Fig. S3). R2A is a medium that contains low concentrations (0.05%) of several nutrients (yeast extract, peptone, casamino acids, glucose, and soluble starch), initially formulated to enumerate bacteria from potable water [16], and frequently used for the growth of bacteria from natural oligotrophic environments.

In addition to its fundamental role within central carbon, metabolism pyruvate is an effective scavenger of reactive oxygen species [11]. In the presence of hydrogen peroxide, pyruvic acid undergoes nonenzymatic decarboxylation to form acetate, $CO_2$, and $H_2O$, a typical reaction of α-keto carboxylic acids [9]. A recent study shows that pyruvate has a critical role in protection from $H_2O_2$ damage for pathogenic spirochetes *B. burgdorferi* and *L. interrogans* [10]. There are also numerous reports pointing to the positive effect that the incorporation of pyruvate has on the cultivation of stressed bacterial cells [17].

Although it has long been known that the tolerance of microaerophilic bacteria to oxygen is largely affected by the composition of the growth medium, the stimulatory effect of pyruvate in streak plates has not been reported before. It resembles the effect noted when cultures of *Campylobacter fetus* were amended with norepinephrine. This drug favored the incorporation of iron required as cofactor by enzymes that protect against oxidative stress [18].

## Comparison of protein expression profiles of planktonic and aggregated growth using 2-D PAGE and mass spectrometry

Global protein-expression profiles of *Sediminibacterium* sp. C3 under aggregated mode of growth (pyruvate added) and planktonic growth (growth without pyruvate) were compared using 2-D protein gel electrophoresis. Proteins were resolved on pI range strips 4–7. About 350 spots with a molecular mass range of 10–100 kDa were detected (Fig. 2a). Ten spots representing nine unique proteins were significantly up-regulated during planktonic growth (Table 1). Only two proteins increased significantly their expression during aggregated growth in comparison to planktonic growth (Fig. 2a).

The most notable finding in the proteomic analysis was the up-regulation of proteins related to stress under planktonic growth conditions. Among these, two isoforms of a protein belonging to the universal stress family (UspA), resolved as two different spots (number 260 and 261) (Fig. 2b and Table 1), ranked among the three most up-regulated spots. Moreover, one of the isoforms (spot number 261) was not detected in any of the four replicates of aggregated growth (Table 1).

*Sediminibacterium* sp. C3 genome [8] has three genes belonging to the Usp family, all classified as UspA. This family of small cytoplasmatic proteins is conserved in bacteria, archaea, fungi, protozoa, and plants [19]. Even though their biological and biochemical functions are not well known, the expression of the UspA superfamily of proteins is largely induced by a variety of environmental disturbances, including oxidative stress. The direct involvement of UspA and its orthologs in the oxidative stress response and survival under both *in vitro* and *in vivo* growth conditions has been demonstrated in *uspA* mutants of a number of bacteria,

**Table 1.** Proteins significantly up-regulated during planktonic (A) and aggregated (B) growth of *Sediminibacterium* sp. C3.

| | | | Theoretical[a] | | Gel | | Mascot values[b] | | |
|---|---|---|---|---|---|---|---|---|---|
| Match ID | Fold change | Protein identity | MW (kDa) | pI | MW (kDa) | pI | score | % coverage | expect |
| A | | | | | | | | | |
| 261 | – | universal stress family protein | 31.5 | 4.85 | 29 | 5.0 | 106 | 22 | 7.60E-08 |
| 262 | 11.8 | hydrolase CocE/NonD family protein | 70.7 | 8.26 | 74 | 6.6 | 96 | 19 | 7.80E-07 |
| 260 | 5.9 | universal stress family protein | 31.5 | 4.85 | 28 | 4.9 | 183 | 22 | 1.50E-15 |
| 305 | 3.9 | putative uncharacterized protein | 28.7 | 5.54 | 30 | 5.9 | 69 | 33 | 3.60E-04 |
| 282 | 2.0 | thioredoxin-disulfide reductase | 34.1 | 5.36 | 32 | 5.5 | 120 | 34 | 3.00E-09 |
| 278 | 1.8 | hypothetical protein | 52.4 | 8.02 | 56 | 6.7 | 171 | 22 | 2.40E-14 |
| 17 | 1.6 | protein Cj1172c (reg transcrip) | 26.7 | 5.38 | 28 | 5.9 | 57 | 24 | 5.80E-03 |
| 64 | 1.5 | DNA polymerase III, beta subunit | 41.4 | 5.12 | 40 | 5.4 | 278 | 45 | 4.80E-25 |
| 148 | 1.4 | SAICAR synthetase family protein | 35.3 | 4.93 | 28 | 5.1 | 270 | 67 | 3.00E-24 |
| 57 | 1.2 | 4-hydroxyphenylpyruvate dioxygenase | 43.5 | 5.02 | 39 | 5.2 | 201 | 46 | 2.40E-17 |
| B | | | | | | | | | |
| 132 | 2.5 | 30S ribosomal protein S1 | 70.1 | 5.22 | 71 | 5.3 | 203 | 29 | 1.50E-17 |
| 89 | 2.0 | ATP synthase F1, beta subunit | 53.8 | 4.98 | 52 | 5.2 | 76 | 29 | 8.50E-05 |

[a]Theoretical MW and pI were obtained from the *Sediminabaterium* sp C3 annotated draft genome [11] used as an in-house database.
[b]Spectra from MS analysis were compared to the *Sediminabaterium* sp C3 annotated draft genome [11] using the MASCOT software. Only proteins identified with Mascot scores >55 were recorded.

including *Escherichia coli* [20] and the pathogenic *Listeria monocytogenes* [21].

Quantitative RT-PCR analysis indicates that differences in the expression of Usp gene between planktonic and aggregated growth were largest during late exponential phase (Fig. 3), confirming that differences in transcription of Usp is affected by nutrient depletion.

The expression of a thioredoxin-disulfide reductase was also significantly higher under planktonic growth (Table 1). This protein is an ortholog of a transcriptional regulator of the microaerophilic food-borne pathogen *Campylobacter jejuni* (Cj1172c), which has been implicated in the response to nitrosative stress, together with other proteins involved in oxidative stress tolerance [22]. Thioredoxins are ubiquitous proteins that derive their name from their ability to catalyze the reduction of disulfide bridges. This activity can restore the function of oxidized proteins under stress conditions [23]. In addition, thioredoxins carry out other activities that play other important functions against oxidative stress, including peroxide hydrogen reduction, singlet oxygen quencher, and hydroxyl radical scavenger [23].

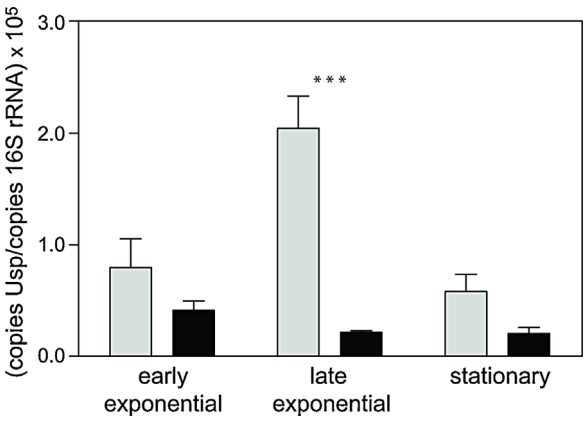


**Figure 3.** Relative expression of USP gene under planktonic growth (gray bars) and aggregate growth (black bars) at different growth phases, determined as the ratio of abundance of target gene to that of 16S rRNA of *Sediminibacterium*. Data are mean ± sem, $n = 6$. Two way ANOVA followed by a Bonferroni post hoc test were used to determine statistical significance, with triple asterisk indicating $p < 0.001$.

The other protein up-regulated under planktonic growth was the CocE/NonD hydrolase (a protein belonging to a family of aminopeptidases). The only two proteins that increased significantly their expression during aggregated growth were 30S ribosomal protein S1 and ATP synthase F1 beta subunit, involved in RNA translation and ATP synthesis respectively (Table 1 and Fig. 2).

We argue that the addition of pyruvate to the culture media protects *Sediminibacterium* cells against oxidative stress. The mechanism by which pyruvate protects against *Sediminibacterium* sp. C3 stress has not been established. It has been postulated that intracellular pyruvate may protect against oxidative stress by regulating the enzymatic pathways involving synthesis in mammalian cells [11, 24]. Interestingly, a recent report demonstrated that hydrogen peroxide stress in *Pseudomonas fluorescens* provoked a metabolic reprogramming that enhanced the production of pyruvate [25].

Pyruvate also induces acid resistance in bacteria by the expression of the small noncoding RNAs, which in turn activates expression of RpoS [26].

For the majority of environmental and pathogenic bacteria, differential gene expression between planktonic and sessile cells is evidenced by the higher resistance to external factors, such as antibiotics, and the increased number of stress-related genes that are associated with the latter form of growth [27–29]. Our results obtained in *Sediminibacterium* differ in that proteins related to stress were found to be upregulated during planktonic growth, resembling the response of marine bacteria to nutrient starvation stress [30].

There are other examples in nature of bacteria that adopt sessile lifestyles within microaerophilic niches and switch to free living growth under aerobic conditions. That is the case of the iron oxidizing bacteria *Leptospirillum* spp. in their natural acidic environment, which alternate between the biofilm and planktonic lifestyles by activating two different metabolic pathways to obtain energy for growth [31].

### Concluding remarks

*Sediminibacterium* sp. C3 can grow free in suspension or attached to surfaces and biofilms. According to our results, the adoption of either type of growth might be governed by the nutrient concentration and or redox conditions of the environment. Microaerophily is a widespread trait of many bacteria from several environments, including subsurface sediments, soil, freshwater, and activated sludge. However, the underlying mechanism behind the common occurrence of microaerophilic growth that appears only restricted to low-substrate media is still not well understood. We found that the addition of pyruvate led to aggregated growth of *Sediminibacterium* sp. C3, mimicking the effect of oxygen scavengers present in high nutrient rich media. Whereas the protective effect of pyruvate against oxidative stress is well known, this is the first report suggesting a role for pyruvate on the autoaggregation of bacteria.

In this work, we show that *Sediminibaterium* sp. C3 prefers the microaerophilic conditions of aggregated growth, but is able to sustain the stress of high dissolved oxygen conditions until it can regain a more favorable environmental niche.

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## Conflict of interest

The authors have no conflicts of interest to declare.

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