

# High-Throughput Monitoring of Bacterial Growth at Elevated Hydrostatic Pressure on the FLUOstar OPTIMA

S. Lucas Black<sup>1,2</sup>, F. Bruce Ward<sup>1</sup> and Rosalind J. Allen<sup>2</sup>

<sup>1</sup>Institute of Cell Biology, University of Edinburgh, UK, <sup>2</sup>SUPA, School of Physics and Astronomy, University of Edinburgh, UK

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- Development of a microplate sealing technique suitable for growth of microorganisms at high hydrostatic pressure
- High-throughput monitoring of growth of the deep sea bacterium *Photobacterium profundum* SS9
- The measured growth rates are dependent on both the hydrostatic and osmotic pressure

## Introduction

Microorganisms display an astonishing ability to survive and proliferate under extreme environmental conditions, including high and low temperatures, high acidity or alkalinity, high salt concentrations, and high hydrostatic pressure.<sup>1</sup> The physiological and biochemical origins of these capabilities are in many cases poorly understood. Although such extreme environments may seem remote from our daily lives, achieving a better understanding of how these extreme microorganisms function is important both from a scientific and biotechnological point of view. From a fundamental point of view, the biochemistry of these organisms sheds light on the limits of operation of the key molecular mechanisms of life. Extremophiles are also believed to be crucial players in the evolutionary tree, since life probably evolved under conditions much more extreme than those that prevail on Earth today. Technologically, extremophiles have the potential to provide new products for molecular biology and other biotech areas.

Our work focuses on the deep sea bacterium *Photobacterium profundum* SS9. This  $\gamma$ -proteobacterium is a close relative of *Vibrio cholerae*. *P. profundum* SS9 was isolated in 1984 from the Sulu Sea at a depth of 2.5 km,<sup>2</sup> and is a piezophile: it grows better at elevated hydrostatic pressure than at atmospheric pressure. *P. profundum* SS9 can grow at pressures ranging from 0.1 MPa to 90 MPa, with an optimal growth pressure of 28 MPa.<sup>2</sup> This bacterium is an ideal organism for studying life at high pressure, because its genome sequence has been determined<sup>3</sup> and a range of genetic tools have been developed, making it relatively easy to manipulate in the laboratory. Like most marine organisms, *P. profundum* SS9 has a requirement for NaCl. It has been observed that the physiological effects of increased hydrostatic pressure are similar to those of osmotic pressure (increased salt). For example, *P. profundum* SS9 produces a similar range of intracellular osmolytes in response to both salt and hydrostatic pressure.<sup>4</sup> The aim of our work is to understand better this intriguing similarity.



Fig. 1: BMG LABTECH's multimode plate reader FLUOstar OPTIMA



Fig. 2: The 3 litre pressure vessel used in this study

This study requires us to grow *P. profundum* SS9 under a range of conditions of hydrostatic and osmotic pressure in the lab. Microplate readers such as the FLUOstar OPTIMA (Figure 1) are extremely valuable for such high-throughput growth studies, as they allow fast and reliable measurements to be achieved much less labour intensively than using traditional methods, and allow much better statistics to be obtained through the use of multiple replicates. Growth of microorganisms is monitored by measuring the absorbance at 600 nm ( $A_{600}$ ) in the plate reader over time. However, the use of a microplate reader for growth under hydrostatic pressure provides unusual challenges. Growth of microorganisms at pressure is achieved using a pressure vessel. The steel pressure vessel used in this work is pictured in Figure 2; it has a capacity of 3 litres and is filled with liquid water which is pressurized. The vessel can accommodate up to 8 standard sized microplates. These microplates must be sealed with a film, attached by a strong adhesive, to prevent leakage in the pressure vessel. The sealing film must be flexible to allow pressure to be transmitted to the well contents. The wells must not contain any air, as gas pockets in the wells will be compressed on pressurization, causing the seal to stretch and break. Furthermore, the seal must be transparent to allow absorbance measurements, and must be non-toxic to the microorganisms under study. We have developed a method for sealing microplates that overcomes these challenges. In this application note, we report preliminary results using this method in combination with the FLUOstar OPTIMA.

## Materials and Methods

### Bacterial Strains and Media

*Photobacterium profundum* SS9 was obtained from Prof. Doug Bartlett (Scripps Institute of Oceanography)<sup>3</sup> and cultured in modified MOPS Minimal (MM) Media. The MM media was made according to Neidhardt *et al.*<sup>5</sup> with a final concentration of 40 mM MOPS, 25 mM Glucose, 9.5 mM  $\text{NH}_4\text{Cl}$ , 4 mM Tricine, 1.32 mM  $\text{K}_2\text{HPO}_4$ , 0.525 mM  $\text{MgCl}_2$ , 0.276 mM  $\text{K}_2\text{SO}_4$ , 0.05 mM  $\text{CaCl}_2$ , 0.01 mM  $\text{FeSO}_4$  and NaCl concentrations ranging from 100 mM to 700 mM. In addition, trace elements and vitamin solutions are added, along with 0.1% casamino acids.

## Preparation of Pressure Proof 96-well Plates

Each well of a Greiner Bio-One flat bottom 96-well plate was filled with 382  $\mu\text{L}$  liquid media. The plate was then sealed with a commercial PCR film, ensuring that air bubbles were not trapped beneath the film. Once the film was in place, a layer of araldite epoxy resin was spread around the edge of the film and allowed to set. This is necessary to prevent leakage in the pressure vessel.

## Culturing Bacteria at High Pressure and Calculation of Growth Rate

High pressure growth at 28 MPa was carried out in a 3 litre pressure vessel with a temperature controlled water jacket set to 15°C. Pressure was generated by a hand pump. To make a measurement of  $A_{600}$ , the microplates were depressurized, dried and  $A_{600}$  was measured in the FLUOstar OPTIMA, without removing the seals. Measurements were made at 3 hour intervals for 81 hours.

Each experiment was performed using three identical 96-well microplates. Each plate contained 11 replicates of 7 different NaCl concentrations. For each NaCl concentration, a blank well contained media alone. The final 12 wells per plate were made up of 4 replicates of 3 crystal violet solutions of known  $A_{600}$  ranging from 0.1 to 1.5.

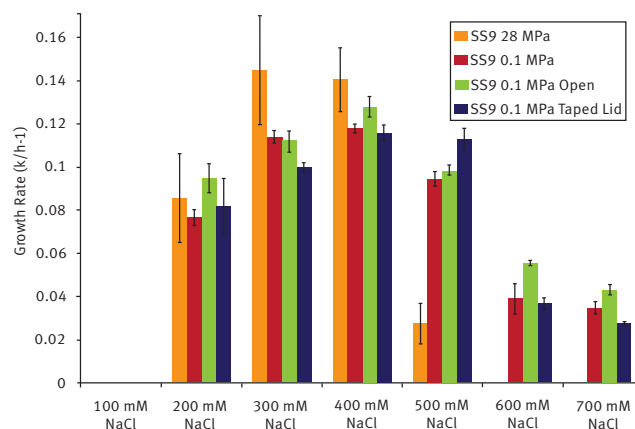
Measurements at different time points were merged using the MARS data analysis software from BMG LABTECH. This data was then exported into Microsoft Excel. During the experiment, the  $A_{600}$  of our blank wells changed over time: this is because the PCR film reacts slowly with the culture medium and becomes slightly opaque. To correct for this effect, we subtracted from each raw data point the measured  $A_{600}$  for the blank well (at that NaCl concentration), measured at the same time. This method of blanking was tested using the 12 wells per plate containing crystal violet solutions of known  $A_{600}$ ; the measured  $A_{600}$  increased with time in a linear fashion. These calibration plots were used to verify the accuracy of the blanked  $A_{600}$  readings for the bacterial samples.

From the corrected  $A_{600}$  data, we were able to determine the growth rate of *P. profundum* SS9 under a range of NaCl concentrations, by taking the slope of a plot of  $\log(A_{600})$  versus time, for the exponential part of the growth curve.

## Results and Discussion

The experiment was carried out at 28 MPa (optimal growth pressure for *P. profundum* SS9) and at 0.1 MPa (atmospheric pressure). We also carried out two control experiments. In the first control, the microplate was not sealed and growth was monitored at atmospheric pressure, with the aim of determining whether oxygen availability in the sealed plates inhibited the growth of *P. profundum* SS9. In the second control, also at atmospheric pressure, a standard polystyrene microplate lid was used and gas exchange with the wells was prevented by taping the lid in an anaerobic chamber. This should result in the same growth rate as our sealed microplate method (at atmospheric pressure) and thus acts as a control for our method.

Our results are shown in Figure 3. Hydrostatic pressure has a marked effect on the range of NaCl concentration at which *P. profundum* SS9 is able to grow. When the hydrostatic pressure was increased from 0.1 MPa to 28 MPa, growth of *P. profundum* SS9 was largely unaffected at intermediate salt concentrations (200-400 mM NaCl), but growth was strongly inhibited at higher NaCl concentration, compared to the results at 0.1 MPa. The optimum NaCl concentration was changed only slightly on changing the hydrostatic pressure. Interestingly, at both 0.1 MPa and 28 MPa there was a dramatic drop-off in growth at NaCl concentrations below 200 mM. The position of this threshold appears to be independent of hydrostatic pressure.



**Fig. 3:** Measured growth rates, averaged over 33 replicates, for a range of NaCl concentrations, for sealed plates at 0.1 MPa and 28 MPa, for open plates at 0.1 MPa and for plates with taped lids at 0.1 MPa.

These preliminary observations indicate a clear coupling between the response to hydrostatic and osmotic pressure in this bacterium. This coupling will be investigated in detail in further work.

Comparing the results of our control experiment with the open plate to those for the sealed plate at 0.1 MPa, we conclude that the effects of oxygen availability are minor, although some decrease in growth in the anaerobic plates may be discernable at the highest NaCl concentrations. We also observed good agreement between the results with the taped lid and the sealed plate, at 0.1 MPa, confirming the reliability of our method for measuring growth rates.

## Conclusion

In this report, we have demonstrated that the FLUOstar OPTIMA is well suited for high-throughput assays of microbial growth, even under extreme conditions such as high hydrostatic pressure. We have described preliminary tests of a method for pressure-resistant sealing of 96-well microplates. Further results with this method, for several deep-sea bacterial strains, will be described in a forthcoming publication.

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Germany: **BMG LABTECH GmbH** Tel: **+49 781 96968-0**

Australia: BMG LABTECH Pty. Ltd. Tel: +61 3 59734744  
France: BMG LABTECH SARL Tel: +33 1 48 86 20 20  
Japan: BMG LABTECH JAPAN Ltd. Tel: +81 48 647 7217  
UK: BMG LABTECH Ltd. Tel: +44 1296 336650  
USA: BMG LABTECH Inc. Tel: +1 919 806 1735

Internet: **www.bmglabtech.com** **info@bmglabtech.com**