Using Ogi3 to maintain bacterial cultures for improved reproducibility of infection studies

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Abstract

Reproducibility is a major hurdle in *in vitro* bacterial infection models, partly due to variability in the state of bacterial cultures across experiments. Here, we demonstrate that using the Ogibiotec bioreactor Ogi3 not only improves reproducibility but also saves time, enabling scientists to focus on their research questions. *E. coli* bacteria were grown in the Ogi3 in turbidostat mode at a defined optical density (OD), by maintaining the culture at an approximately constant OD by diluting it whenever it exceeded the set OD, and used to inoculate a monolayer of bladder carcinoma cells. We show that this approach ensured consistent bacterial density at the point of inoculation and reduced inter-experimental variability by maintaining steady-state growth conditions and stable bacterial physiology.

Introduction

In vitro models of infection are an important tool to study bacterial infections (Flores et al., 2023; Jafari & Rohn, 2023; Moule et al., 2016; Newman et al., 2022; Sharma et al., 2021; Thumbikat et al., 2009). In a typical experiment, bacteria are introduced to a cell culture at a defined ratio of bacteria to host cells. This ratio is known as the specific multiplicity of infection (MOI). Because counting bacteria before each experiment is laborious, the MOI is often inferred from the OD of the inoculum culture. This approach requires establishing a standard curve relating OD to bacterial density (Colony Forming Unit per ml, CFU/ml) by plating cultures of known OD (Rutten et al., 2019; Van Alst et al., 2023). The standard curve depends not only on the particular strain and the medium used, but also on the bacterial growth phase (Scott & Hwa, 2011). For example, fast-growing bacteria are larger than slow-growing ones, which affects how they scatter light in OD measurements. Hence, the same OD can correspond to different concentrations of bacterial cells, depending on growth conditions, and not accounting for this can lead to incorrect estimation of MOIs. Moreover, bacterial physiological state affects their adhesion to mammalian cells and cell infection rates (Ha & Jin, 2001; Lee & Falkow, 1990; Mouslim & Hughes, 2014). Achieving reproducible results in infection experiments, therefore, requires using bacterial cultures that are in a consistent physiological state.

Unfortunately, the nature of infection experiments frustrates the fulfilment of these needs. Scientists often prepare an overnight bacterial culture and either dilute it down to the desired OD immediately before the infection experiment, or inoculate a fresh culture in the morning and grow it to the target OD. The former approach introduces variability in bacterial density between experiments, while the latter extends the duration of an already long procedure.

Here, we demonstrate how to overcome these technical challenges using Ogibiotec's Ogi3 bioreactor. We ran the bioreactor in turbidostat mode to maintain cultures of commensal *E. coli* overnight at an OD of 0.25, corresponding to an early exponential phase. We then used the bacteria from the turbidostat culture to perform a bacterial colonization assay on a monolayer of bladder carcinoma cells. We show that by using this method, the number of bacteria able to colonize the cells was highly reproducible.

Materials and methods

Ogi3 bioreactor

Ogi3 is a modular bioreactor developed and manufactured by Ogibiotec (Edinburgh, UK). The experiment described below uses only two modules: the main unit and the liquid control module. The main unit has four independently-controlled flask holders, each equipped with OD and temperature sensors, a heater, and a magnetic stirer. Each flask can hold between 15 and 25 ml of bacterial culture. The liquid control module is connected to the main unit and can add media to the flasks as well as remove the surplus culture using a set of 8 peristaltic pumps (two per flask).

Bacterial culture

A culture of *E. coli* (MG1655, PtetO1-mKate2 PsfiA-mGFP, eSJR206; Jaramillo-Riveri et al. (2022)) was prepared in LB. Before use, the tubing was rinsed with 70% ethanol for 1 minute, followed by 2 minutes with sterile, deionised water using the pump cleaning program. A temperature probe was wiped with 70% ethanol and connected to the system. A media reservoir containing 500 ml LB was connected to the source tubing, and an empty 500 ml waste bottle was connected to the drain tubing. A reactor flask was filled with 15 ml LB and a sterilized magnetic stir bar. The medium was inoculated from a bacterial glycerol stock, and the flask was closed with a stopper. Source tubing from the LB reservoir was connected to the flask, and drain tubing was connected from the flask to the waste reservoir. The temperature probe was inserted into the culture to around 5 mm below the surface of the LB. The system was then run in turbidostat mode, in which the culture was diluted automatically with fresh medium whenever its optical density (measured at 3 min intervals) exceeded a set threshold of OD=0.25. See Appendix 0.1 for all Ogi3 parameters. Figure 1 shows an example curve of OD versus time from a single turbidostat run. Following inoculation, the optical density increases until it crosses the threshold OD for the first time. Then, the system adds a small volume of the medium, simultaneously removing surplus volume by running the inlet and outlet pumps for a short time. This causes the OD to decrease below threshold OD before growth causes it to increase again. The process is then repeated for the duration of the culture experiment. The culture was carried out overnight and used the next morning for the colonization assay.

Mammalian cell culture

5637 bladder carcinoma cells (ATCC, HTB-9) were grown in DMEM (Gibco, 41966-029) supplemented with 10% FBS (Gibco, 10270-106) in T25 flasks at 37°C and 5% CO_2 until 80% confluency. For passaging, the cells were washed with PBS and trypsinised for 8 minutes with 0.05% trypsin - EDTA (Gibco, 15400-054).

Colonization CFU assay

Bladder carcinoma cells were grown on 200 µl collagen in 24-well plates until confluent (approximately 2.4 x 10^5 cells). Collagen was prepared by mixing 445 µl of type I rat tail collagen (3 mg/ml, Corning, 354236) with 55 µl of 2:1 10x DMEM (Sigma, D2429) containing $NaHCO_3$: 0.4 M NaOH, and further neutralised by adding 20 µl of 0.4 M NaOH.

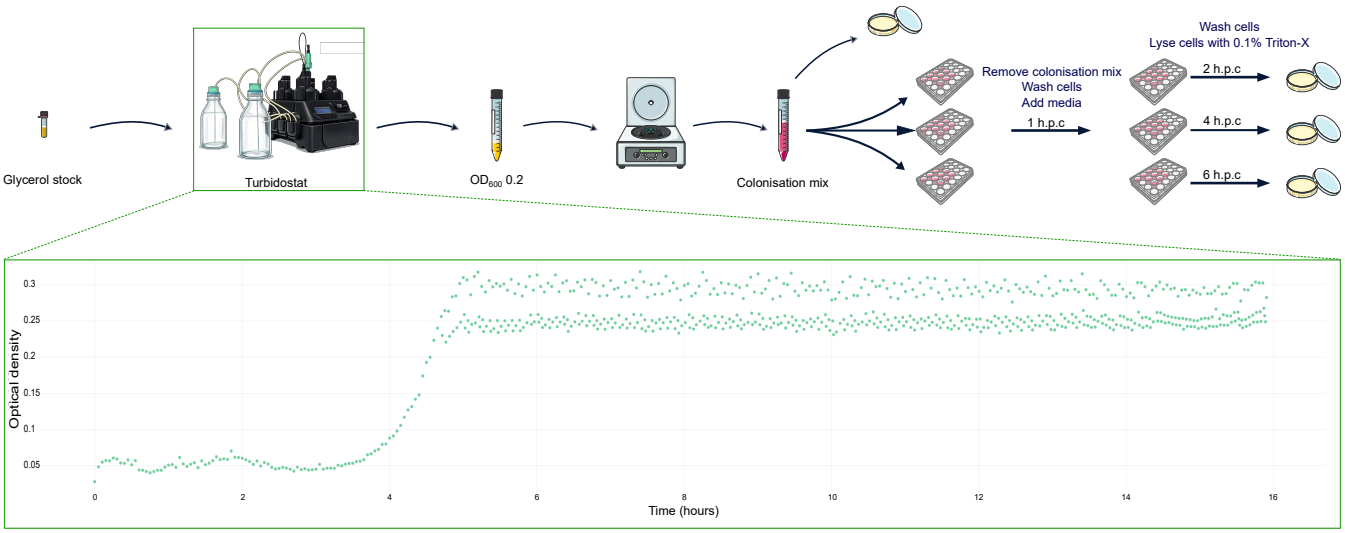

Figure 1: Workflow of the colonization CFU assay. An overnight *E. coli* culture was inoculated in LB from a glycerol stock and incubated in an Ogi3 bioreactor run in turbidostat mode. The bioreactor maintained the culture at an approximately constant OD by diluting it whenever the OD exceeded 0.25. OD fluctuations visible in the plot are caused by a combination of fast growth and infrequent sampling, which causes the system to over/undershoot. Immediately before the infection experiment, a specific volume of culture was spun down and resuspended in the cell culture medium to obtain colonization mixes. These mixes contain a defined number of bacteria in the early exponential growth phase. The colonization mixes were added to a monolayer of 5637 bladder carcinoma cells and incubated for 1 hour. In parallel, a sample of the initial colonization mix was diluted and spotted on LB agar to confirm the density of bacteria. After a one-hour incubation, the mammalian cells were washed with PBS, and fresh media was added. The cells were again washed 2, 4, and 6 hours post the colonization (h.p.c), and lysed with Triton-X. Lysates were diluted and plated on LB agar plates to obtain the number of bacteria that adhered to mammalian cells.

The colonization mix was prepared as follows: 2.4 ml of the turbidostat culture (4 x 10^7 cfu/ml, determined using an OD-to-CFU calibration curve obtained prior to the experiment) was spun down (10 minutes, 4000 rpm) and resuspended in 8 ml DMEM, DMEM-25% urine, or DMEM-50% urine, yielding a colonization mix of 1.2 x 10^7 CFU/ml. Before colonization, monolayers were washed once with 1 ml PBS. 200 µl (2.4 x 10^6 bacteria, equivalent to MOI=10) of the colonization mix was added to all wells except the control well, to which 200 µl of bacteria-free medium was added. Plates were incubated for 1 hour at 37°C with 5% CO_2. While the plates were incubating, the remaining colonization mix was serially diluted ten-fold in PBS to 10^{-7} in a 96-well plate by transferring 20 µl into 180 µl PBS at each dilution step. 10 µl of each dilution was spotted in triplicate onto LB agar plates to determine the CFU in the inoculum. After the colonization, the inoculum was aspirated, and cells were washed twice with 1 ml PBS per well. Fresh medium was then added at 500 µl per well. Plates were incubated for a further 1 hour at 37°C with 5% CO_2. The content of each well was aspirated 2, 4, and 6 hours post colonization, and the monolayers were washed twice with 1 ml PBS per well. Cells were lysed by adding 200 µl of 0.1% Triton X-100 per well and incubating for 5 min at room temperature. Lysates were serially diluted ten-fold in PBS in a 96-well plate by transferring 20 µl lysate into 180 µl PBS. 10 µl of each dilution was spotted in triplicate onto LB agar plates and incubated at 37 °C overnight. Colonies were counted, and CFU per well were calculated based on the dilution factors and spotted volumes. The process is depicted in Figure 1.

Results

We colonized a urothelial cell line with *E. coli* in different concentrations of urine to replicate different environments bacteria may experience in the bladder. We used three different colonization times: 2, 4, and 6 hours, and three different cell culture media: DMEM, DMEM-25% urine, and DMEM-50% urine. We aimed to add 2.4 x 10^6 bacteria per well to obtain MOI=10. Figure 2 shows the actual number of bacteria obtained from plating diluted inoculation mixes. The inocula remained very consistent; the slightly higher inoculum in DMEM was caused by the bacteria replicating in this rich medium during the experimental procedure.

Figure 3 shows the results of colonization assays. 2 hours post colonization, between 10^4 and 10^6 bacteria had adhered to and grown on the bladder carcinoma cells. The number of adhered bacteria was consistent across experimental repeats. This can be explained by low variability in the number of bacteria being added. The same growth trends were observed across experimental repeats and experimental conditions. As the Ogi3 maintains the bacteria at an OD of 0.25, the bacteria are in the early exponential phase when added to the mammalian cells, which helps to maintain their consistent physiological state.

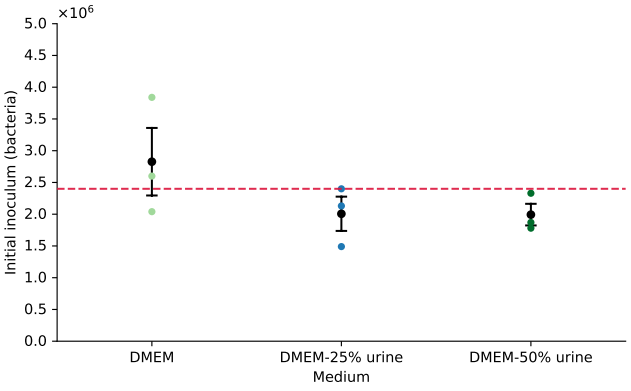

Figure 2: The Ogi3 provides consistent initial inocula. The initial inoculum, obtained by maintaining an *E. coli* culture at OD 0.25 with the Ogi3 in turbidostat mode, spinning down and resuspending in the medium of interest before the start of the colonization experiment. Error bars represent the standard error of the mean. The red dotted line is the target inoculum.

Conclusion

Consistent bacterial inocula are essential for achieving reproducible results in infection studies. However, current inoculum preparation protocols are either time-consuming or do not ensure that the bacteria used are in a consistent physiological state. To reduce phenotypic heterogeneity of the bacterial population, it is best to culture bacteria in steady-state exponential growth conditions, although this approach is labour-intensive when

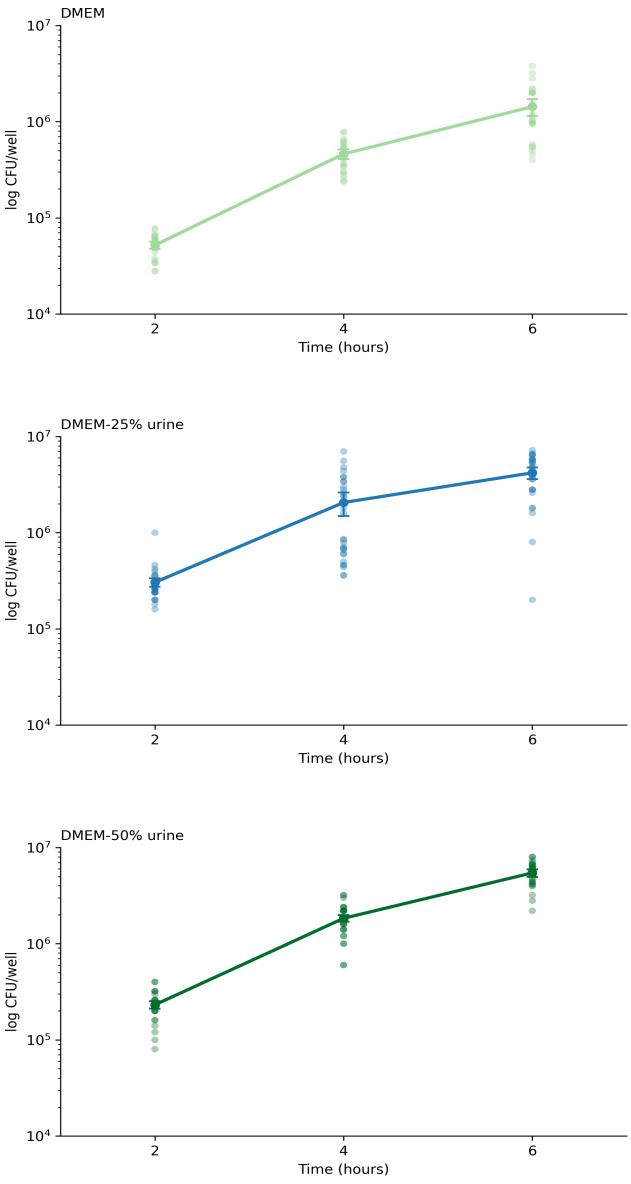

Figure 3: The Ogi3-maintained bacterial culture lead to consistent growth patterns across experimental replicates. The plots show CFU/well at 2, 4, and 6 hours post colonization in DMEM, DMEM-25% urine, and DMEM-50% urine.

performed manually. Here, we have demonstrated that the process can be effectively implemented using the Ogi3 bioreactor run in the turbidostat mode.

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Author contributions

L.G., M.M., M.E.K., and J.A.D. designed the experiments, and L.G. performed the experiments. L.G. wrote the manuscript, and M.M., M.E.K., G.B., B.W., and J.A.D. edited the manuscript.

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Appendices

0.1 Ogi3 Experimental Parameters

Table 1: Ogi3 experimental settings used in this study.

Parameter	Experimental setting
Light source	LED
Wavelength	605 nm
Ambient temperature	20 °C
Bioreactor temperature	37 °C
Stirring speed	4000 rpm
OD sampling interval	3 minutes

0.2 Ordering and Contact Information

Chemostat/Turbidostat configuration:

- Ogi3 Bioreactor core module – Cat# OGI3-1010
- Ogi3 Liquid control module – Cat# OGI3-1011

Additional modules available:

- pH Module – OGI3-1012
- Dissolved Oxygen Module – OGI3-1008
- Sparging Module – OGI3-1013
- Fluorescence Module – OGI3-1014

System enquiries should be sent to info@ogibiotec.com or telephone: +44(0)131 445 6116