Constitutively expressed fluorescent proteins allow to track bacterial growth and to determine relative fitness of bacteria in mixed cultures

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Abstract

Bacterial growth is classically assessed by measuring increase in optical density of pure cultures in shaken liquid media. Measuring growth using optical density has severe limitations when studying multistrain interactions as it is not possible to measure the growth of individual strains within mixed cultures. Here we demonstrate that constitutively expressed fluorescent proteins can be used to track growth of individual strains in liquid cultures. Fluorescence measurements highly correlated with optical density measurements. This allowed us to assess bacterial growth not only in pure cultures, but also in mixed bacterial cultures and determine the impact of competitors on a focal strain, thereby assessing relative fitness. Furthermore, we were able to track the growth of two different strains at the same time by using fluorescent proteins with differential excitation and emission wavelengths. Bacterial densities measured by fluorescence yielded more consistent data between technical replicates than optical density measurements. Our setup employs fluorescent microplate readers that allow for high throughput and replication.

Keywords: competition, optical density, mScarlet, sYFP2, high throughput

Introduction

Measurement of bacterial growth in liquid culture is a central paradigm for microbiology. Growth is specific for every bacterial strain and growth rates are determined e.g. as part of every characterisation of novel bacterial species or to describe the impact of mutations on bacterial fitness (Baba *et al.* 2006). Classically, growth is assessed by determining changes in turbidity and absorbance of liquid cultures using turbidity standards such as the McFarland standard (McFarland 1907) or turbidity measurements in a spectrophotometer (Dalgaard *et al.* 1994). These methods can be used to determine growth rates and predict cell numbers, maximal optical density (OD) of individual strains, or whole communities in liquid medium. However, they suffer from limitations as spectrophotometers require bacterial cells to be well mixed without any formed clumps that would increase light scattering or cause heterogeneous turbidity (Stevenson *et al.* 2016). Thus, measuring growth of bacterial species that form aggregates in liquid media can be problematic. Furthermore, it is not possible to determine the growth of more than one bacterial strain in the same culture at the same time since only their combined optical density can be assessed. Alternatively to optical density, fluorescence dyes have been used to determine cell densities, e.g. the addition of the DNA intercalating dye acridine orange has been used to determine cell densities (Guo *et al.* 2017). This is possible by fluorometric analysis of liquid samples. Next to chemical dyes, fluorescent proteins can be detected in fluorometric analysis.

The green fluorescent protein (GFP) has been used in molecular microbiological experiments since the nineties of the last century (Chalfie *et al.* 1994; Bloemberg *et al.* 1997). Since then, many additional fluorescent proteins have been discovered or developed (e.g. (Shaner *et al.* 2004; Goedhart *et al.* 2012)). Many of these new proteins have dramatically different properties such as improved brightness and photostability, but also different spectral excitation and emission properties (Shaner, Steinbach and Tsien 2005; Shaner, Patterson and Davidson 2007; Lambert 2019). Using GFP as a proof of concept, it has been demonstrated that constitutively expressed fluorescent proteins can be used to predict growth and colony forming units of *Pseudomonas aeruginosa* (Wilson *et al.* 2018). This encouraged us to pursue a similar approach to predict the growth of bacteria in different liquid media and in co-culture with other differentially-tagged bacteria.

Here we demonstrate the use of different constitutively expressed fluorescence proteins as a means to measure bacterial growth. Furthermore, we make use of proteins with differential excitation and emission spectra (Schlechter *et al.* 2018) to allow the measurement of several populations at the same time. We performed our experiments in 96-well microtiter plates and microtiter plate readers, which allows for high replication and time series monitoring of fluorescent signals and cell growth. By combining these measurements with a series of controls, we demonstrate that our system allows us to determine the strength of interaction and relative fitness of bacterial strains in competition.

Results

Growth of Pe::red in nutrient broth

To highlight the advantage of fluorescence intensity measurements over optical density measurements, we tracked the growth of five replicated Pe::red cultures using optical density and fluorescence intensity in nutrient broth (NB) (Figure 1). The optical density

increases exponentially for ~5 hours and an optical density of 1.2. Afterwards, the culture entered a stationary phase for about 20 hours followed by a rapid decline in optical density. The corresponding fluorescence increases exponentially for ~10 hours and to a fluorescence intensity of 20,000 arbitrary units (a.u.). Afterwards, the rate with which fluorescence intensity increased was slightly reduced, indicating that cell growth decreased. After 20 hours, the fluorescence intensity peaked at about 35,000 a.u.. After a small reduction of fluorescence, indicating cell lyses, the fluorescence increased again till the end of the experiment at 48 hours.
Tracking the optical density shows deviating curves, even though the cultures were seeded from the same original culture and were diluted to the same initial optical density. The deviation was up to 0.25 OD_{600nm} which is more than 10% between the individual cultures. By contrast, the fluorescence of the individual cultures exhibited much lower deviations showing that measuring fluorescence yielded more consistent results. Furthermore, the decrease in optical density after 20 hours is usually interpreted as cell lysis in the classical literature, however, the maintenance of high fluorescence intensity indicated that this is not the case, since cell lysis would also lead to a loss of fluorescence as fluorescent proteins would be denatured. Instead, flock or biofilm formation are a more likely explanation for the drop of optical density.

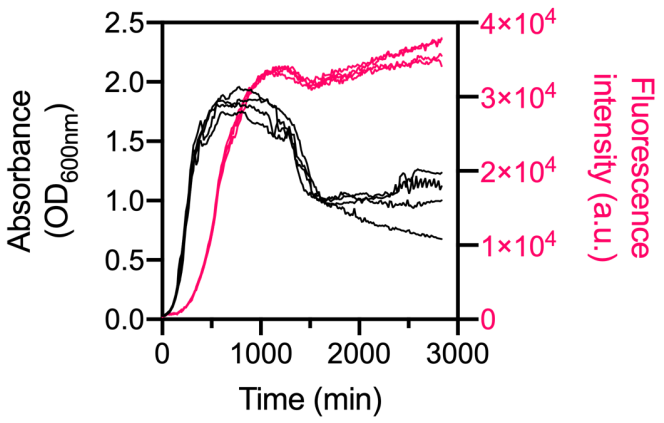

Figure 1. **Example of five cultures of Pe::red grown in NA a 96-well plate. Growth was tracked using optical density (black) and fluorescence intensity (magenta).**

Growth of Pe::red in diluted NB

After inoculation into different concentrations of NB, Pe::red grew up to different final optical densities, while the initial lag phase and increase was similar (Fig. 2A). As expected, the final optical density was directly dependent on the concentration of the NB medium. Depending on the available resources, the different cultures reached their stationary phases at different times and densities. Pe::red grown on 6.75% NB reached its stationary phase after ~2.5 hours while cultures growing on 12.5, 25 and 50%, respectively reached their stationary phase after 5 hours. Cultures growing on higher concentrations of NB reached their stationary phase between 7.5 and 10 hours. It is noteworthy to mention the large standard deviations and irregular curves, especially after the cultures entered their respective stationary phase. This is more pronounced at higher optical densities. After 15 hours, some of the cultures exhibited a small decline in optical density.
For fluorescence intensity measurements, the same cultures exhibited similar increases in fluorescence over time as optical densities at increasing concentrations of NB (Fig. 2B). The increase in fluorescence was lagging behind the optical density increase, likely due to protein maturation rate. The overall ranking of maximal fluorescence was the same as for the optical density measurements. The variability between the replicates was minimal in comparison to the optical density data. The fluorescence data did not exhibit a decline compared to the optical density data and remained stable till the end of the experiment after 20 hours.

Correlating bacterial growth measured using optical density with growth measured using fluorescence

Next, we tested if constitutively-expressed fluorescence signals can be used as a proxy for growth. To that end, we used different measures of the optical density and fluorescence data: the area under the curve (AUC), the maximal value, and the final value for each curve. We fitted these data into linear regression models of fluorescence and optical density to determine the parameters that gave the best fit by comparing the adjusted R^2 and additionally, we determined Pearson's correlations (Table 1, Fig. 3 and Supplemental figure 1). Generally, we observed that all linear regressions fitted the data well with R^2 >0.95. AUC of fluorescence data was the best predictor for every parameter of optical density used (i.e., AUC, maximum value, and final value), resulting in the best fits (R^2 = 0.98) and highest Pearson's correlation values (0.99) (Table 1).

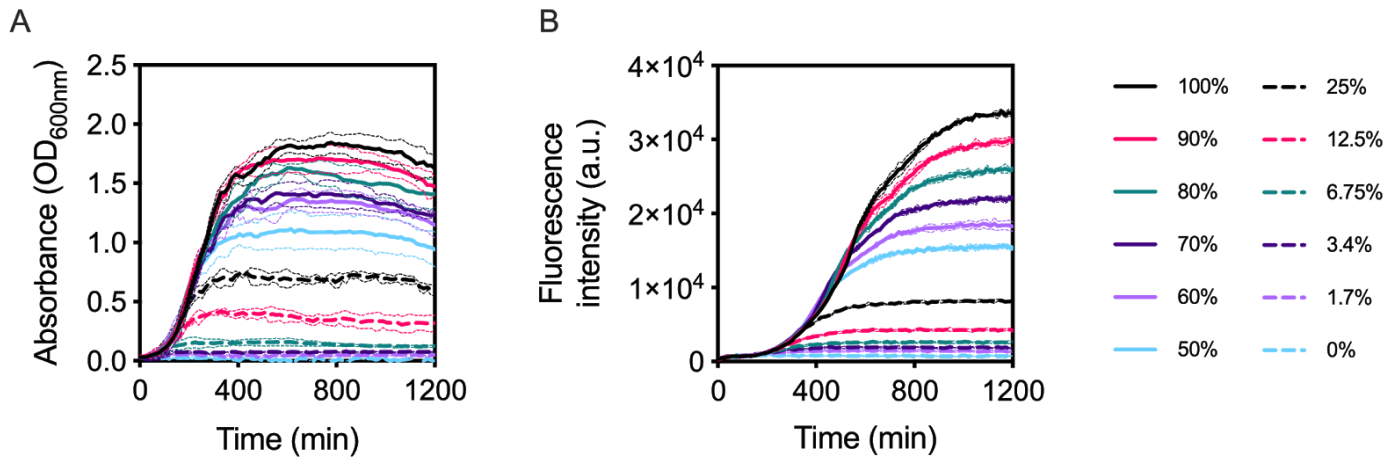

Figure 2. **Growth of Pe::red in different NB concentrations ranging from 0 to 100% in a 96-well plate (see legend on the right) measured by A) optical density at 600 nm or B) constitutively expressed red fluorescent protein as a proxy.**

Consequently, all tested fluorescence signal derived data allow the tracking of growth in liquid cultures and fluorescence can be used instead of OD_{600nm}.

Table 1: **Parameters derived from linear regression models and correlation between fluorescence and absorbance measurements.**

Response Variable	Explanatory Variable	Adjusted R^2	Pearson's correlation
AUC OD	AUC RFU	0.98	0.99
AUC OD	Max RFU	0.95	0.98
AUC OD	Final RFU	0.95	0.97
Max OD	AUC RFU	0.98	0.99
Max OD	Max RFU	0.95	0.98
Max OD	Final RFU	0.95	0.98
Final OD	AUC RFU	0.98	0.99
Final OD	Max RFU	0.96	0.98
Final OD	Final RFU	0.96	0.98

AUC: Area under the curve; Max: Maximum value of dataset; Final: Final value of dataset

Growth of Pe::red and Sp::yellow in presence of different competitors

To determine the impact of different competitors on the growth of Pe::red and Sp::yellow, we inoculated mixed cultures into 96-well plates and covered the plates with a gas permeable foil to avoid evaporation during long-term incubation. Each fluorescently-tagged strain was mixed with their respective wild type conspecific strain (Pe::red vs. PeWT and Sp::yellow vs. SpWT), and also a mix combining both strains (Pe::red vs. Sp::yellow), allowing us to track growth of each strain in parallel. As control, fluorescent strains were grown in monoculture, Their respective fluorescence over time was determined every 10 minutes (Fig. 4 A). As expected when grown without a competitor, Pe::red and Sp::yellow both reached the highest fluorescence intensity and hence the highest cell density (Fig. 4 A, entire lines). When grown against their near-isogenic wild types, the fluorescence did not reach the same high levels depicting the impact of the competitor on the focal strain. Pe::red showed a much more rapid increase in fluorescence compared to Sp::yellow, indicating a higher growth rate. We used the AUC of the growth curves as a proxy for bacteria abundance and to determine bacterial population sizes relative to their respective fluorescent monoculture. The relative AUC of the competition Pe::red vs. PeWT was on average 0.35 when normalised by the AUC of the Pe::red monoculture (Fig. 4 B). The relative AUC of Sp::yellow vs. SpWT normalised by the AUC of the Sp::yellow monoculture was 0.49 and hence Sp::yellow grew to almost exactly half the population density of the monoculture (Fig. 4 C). When competing Pe::red and Sp::yellow, the relative AUC of Pe::red and Sp::yellow was 0.79 and 0.22 when normalised by their respective monocultures (Fig. 4 B and C). Hence, Pe::red had a competitive advantage over Sp::yellow and the Sp::yellow was strongly impacted by the presence of Pe::red.

Discussion

We have designed an experimental platform that allows tracking of bacterial growth in 96-well plates using fluorescence measurement instead of optical density. This allows us to track distinct populations in parallel and we provide an example of a mixed culture of two populations. Fluorescence measurements in plate readers also have the advantage of being less sensitive to cell aggregation than optical density as several measurements of the same culture are taken and averaged, thereby integrating large areas of the sample and accounting for aggregates and less dense areas of the sample. In conjunction with controls, this allows for the measurement of competitions between strains in a high throughput manner. With optimised detector systems such as filters or monochromators and well chosen fluorescent proteins, it is possible to track at least three differently tagged populations without complex experimental controls (e.g. cyan, yellow, and red fluorescent proteins).

We initially performed our experiments with *Pantoea eucalypti*, a fast growing strain that does not form biofilms in shaken liquid culture. This allowed us to compare optical density and fluorescence intensity under ideal conditions. Growth curves of optical density and fluorescence of bacterial cultures do not resemble each other perfectly, which is not surprising since the rate and maturation of fluorescent proteins is a combination of time consuming steps from DNA transcription, mRNA translation and protein maturation before the fluorescence signal can be detected (Leveau and Lindow 2001). This process is however reproducible under controlled growth conditions. Furthermore, others have shown that by using a standard curve, it is possible to predict colony forming units using fluorescence data (Wilson *et al.* 2018). Our experiments have shown that the constitutive expression of fluorescent proteins is suitable to determine bacterial growth in shaking liquid cultures. Every measure that we tested (summarised in Table 1) correlated well with bacterial growth as measured by optical density.

Since growth assessed using fluorescence yields more consistent data than optical density in 96-well plates, it may also be useful in applications such as normalisation of fluorescence intensity of bacterial bioreporters (Tecon, Binggeli and van der Meer 2009). Using our workflow, it is possible to conveniently determine relative fitness of bacteria. Previously, the relative fitness of bacteria has been assessed using shared media reservoirs and filters to separate bacterial populations, which allowed for low throughput investigations of bacterial densities after the competition measured by optical density (Ahn, La and Forney 2006) or, by determining the colony forming units at different sampling times in shared media (e.g. (Ross-Gillespie *et al.* 2007; Friedman, Higgins and Gore 2017)). Both methods have in common that they cannot follow population dynamics at high resolution, such as early or late success or bacterial growth rate in presence of a competing species. By using continuous tracking of bacterial cultures in combination with control experiments that allow for normalisation we are able to infer changes to growth during all phases of an experiment.

To overcome evaporation of medium, which is one of the major drawbacks of 96-well plates that leads to strong edge effects and impacts optical density, we have used hydrophobic, gas-permeable foils to seal 96-well plates. The foils reduced evaporation to negligible amounts over the course of our experiments (up to two weeks, data not shown). At the same time, it also leads to reduced oxygen availability for bacterial growth and limits the ability to measure optical density due to turbidity of the foil and condensation of droplets on the foil. However, since the reduced oxygen availability impacts on the whole plate equally, competition treatments and respective control will be both similarly impacted and the resulting relative fitness can still be assessed.

The experimental system is not without flaws and even though many bacterial strains exhibit negligible autofluorescence in liquid media, some exhibit strong autofluorescence, such as Pseudomonads in minimal media (Remus-Emsermann *et al.* 2016). To be able to accurately assess the growth of strongly autofluorescent bacteria, it is necessary to determine the autofluorescence of the respective parental strain without the fluorescent protein expressed. One clear disadvantage of fluorescent proteins is that many bacterial strains are not yet amenable to genetic modification. However, several recent publications were able to increase the breadths of bacterial recipients that can successfully be manipulated using stable plasmids that do not require antibiotic pressure for medium term maintenance, or (transposon mediated) chromosomal integration (Choi and Schweizer 2006; Schada von Borzyskowski *et al.* 2015; Schlechter *et al.* 2018). Our system should still be interesting for those hard to modify bacteria by competing them against known, fluorescently-tagged

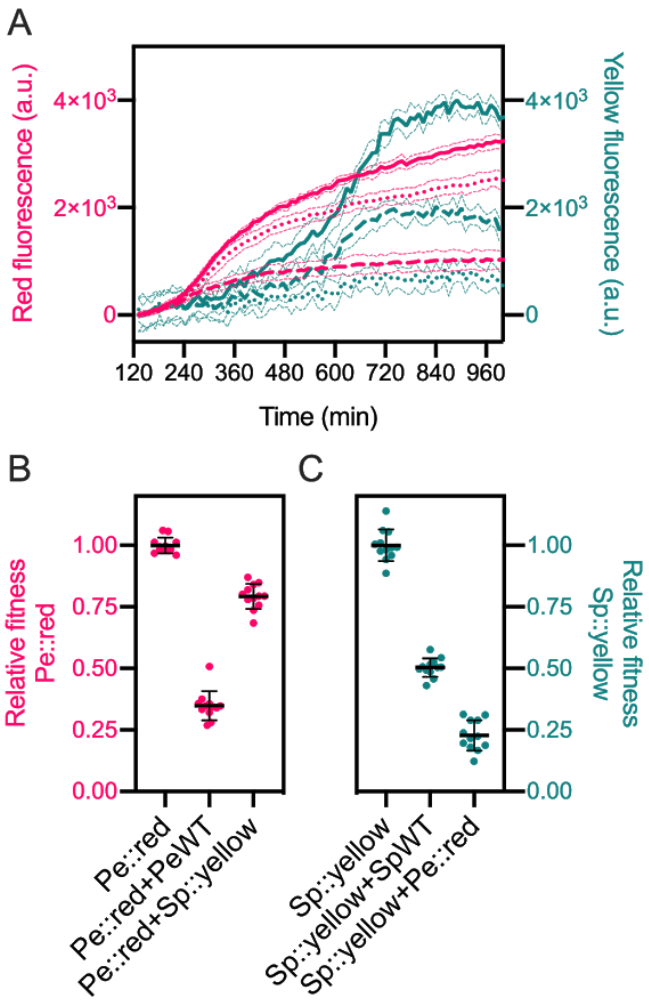

Figure 4. **Competition assay. Fluorescently-tagged bacterial strains were competed in NB and their growth was estimated using fluorescence intensity. The data was background subtracted to normalise against the autofluorescence of the media. A) Magenta: red fluorescence; dark green: yellow fluorescence. Continuous lines represent monocultures of Pe::red or Sp::yellow, respectively. Broken lines represent binary cultures of Pe::red or Sp::yellow with their respective wild types PeWT or SpWT. Dotted lines represent each cultures of Pe::red and Sp::yellow in co-culture. Thin broken lines represent the standard deviation from the mean. B) Relative fitness of Pe::red in monoculture, co-culture with PeWT, and co-culture with Sp::yellow. Fitness determined as the AUC derived from red fluorescence relative to Pe::red in monoculture. C) Relative fitness of Sp::yellow in monoculture, co-culture with SpWT, and co-culture with Pe::red. Relative fitness of Sp::yellow was determined as the AUC derived from yellow fluorescence relative to Sp::yellow in monoculture. The middle line depicts the mean, error bars depict the standard deviation of the mean.**

organisms by determining the impact of the non-tagged organism on the tagged one.

Conclusion

We have designed a convenient experimental platform that tracks bacterial growth by employing constitutively expressed fluorescent proteins. Our platform allows us to track several fluorescence signals simultaneously in a time-resolved manner and in high replications. It yields more robust results than classical turbidity measurements and can be used to determine competition or to perform normalisation of bioreporter experiments

Materials and Methods

Strain and growth conditions

Strains used in this study are listed in Table 2. Bacteria were routinely grown on nutrient broth (NB, HiMedia) or nutrient agar (NA, HiMedia) supplemented with 15 mg L^{-1} gentamicin or tetracycline where appropriate. Broth cultures were incubated at 30°C in a rotary shaker at 200 rpm. Agar plates were incubated at 30°C. The sYFP2 (Kremers *et al.* 2006) expressing strain *Sphingomonas* sp. Fr1::Tn5-mre143 was constructed using the pMRE-Tn5-143 plasmid described previously (Schlechter *et al.* 2018) and the protocol outlined elsewhere (Schlechter and Remus-Emsermann 2019). *Pantoea eucalypti* 299R::Tn7-mre145 expresses the red fluorescent protein mScarlet (Bindels *et al.* 2017). Both fluorescence proteins were driven by the kanamycin promoter.

Table 2: Bacterial strains used in this work

Strain	Short name; Features	Source
Pantoea eucalypti 299R	PeWT	(Remus-Emsermann *et al.* 2013)
Pantoea eucalypti 299R::Tn7-mre145	Pe::red; red fluorescent, gentamicin resistant	(Schlechter *et al.* 2018)
Sphingomonas sp. Fr1	SpWT	(Innerebner, Knief and Vorholt 2011)
Sphingomonas sp. Fr1::Tn5-mre143	Sp::yellow; yellow fluorescent, gentamicin resistant	This study

Plate reader experiments

The 96-well plate was closed with a lid or with a hydrophobic gas permeable membrane 4ti-0516/96 (Brooks Life Sciences, Wotton, UK; gas permeability 0.6 m^{-3} m^{-2} day^{-1} and a water loss of 1 g m^{-2} day^{-1}) and the plate was incubated in a FLUOstar Omega plate reader (BMG Labtech) at 30°C for up to 2 days. The plate was shaken in "meander corner well shaking" mode at 300 rpm between each read. Readings of optical density at 600 nm and fluorescence were measured in bottom optic mode. Fluorescence was measured in a 2 mm diameter circle and the average of 8 measurements per well was recorded. mScarlet fluorescence was excited at 567-587 nm and emission was measured at 613-626 nm. sYFP2 fluorescence was excited at 475-492 nm and emission was measured at 511-550 nm.

Pe::red growth in diluted NB

To grow bacteria Pe::red in flat bottom 96-well plates (COSTAR 96), 10 mL overnight culture were washed twice. To that end, the culture was harvested by centrifugation at 6000 *g* and resuspended using 10 mL 1 x phosphate buffered saline (8 g L^{-1} NaCl, 0.24 g L^{-1} KCl, 1.42 g L^{-1} Na_2HPO_4, 0.24 g L^{-1} KH_2PO_4). Finally, the washed culture was resuspended and diluted to an OD_{600nm} of 0.05. To each well in the 96 well plate, aliquots of 20 µL resuspended culture were added to 180 µL NB or NB diluted to 90, 80, 70, 60, 50, 25, 12.5, 6.75, 3.4, 1.7 or 0%, respectively. The plate was closed with a lid. Optical density at 600 nm and red fluorescence were measured every 15 minutes.

Competition experiments

Bacterial cultures were grown overnight in NB with the appropriate antibiotics. The overnight cultures were harvested at 2000 *g* and resuspended in NB without antibiotics to an OD_{600nm} of between 0.36 and 0.38. Four µL of the diluted cultures were inoculated into 192 µL NB in triplicates to perform the following competition experiments: Pe::red + 4 µL NB, Pe::red + PeWT, , Sp::yellow + 4 µL NB, Sp::yellow + SpWT, Pe::red + Sp::yellow. The 96-well plates were sealed with a hydrophobic gas permeable membrane.

Optical density at 600 nm, red and green/yellow fluorescence were measured every 15 minutes.

Data analysis

Data was analysed using the software Prism 9 (Graphpad, San Diego, CA, USA) and *R* (R Core Team and Others 2013). AUC of the optical density and measured fluorescence was determined using the integrated function in Prism 9. Optical density and fluorescence data were fitted into simple linear regression models using the function "lm" from the *R* package *stats*. Pearson's correlations were determined using the function "cor" from the *R* package *stats*.

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