

Expression of a stable green fluorescent protein mutant in group B streptococcus. Growth, detection and monitoring, with the CLARIOstar®

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- A new recombinant plasmid, pGU2664, stably expresses the highly fluorescent GFPmut3 biomarker in GBS
- The GFPmut3 biomarker can be successfully applied to track the growth of bacteria in liquid media
- CLARIOstar® acquires absorbance, fluorescence and polarized fluorescence to characterize GFPmut3 GBS

Introduction

Streptococcus agalactiae (also known as group B streptococcus [GBS]) is associated with various diseases and infections in humans and animals.¹ These include neonatal disease, sepsis, arthritis, pneumonia, meningitis, skin and soft tissue infections such as urinary tract infection.

Research efforts focused on the mechanisms for host colonization and virulence have mainly utilized microscopy techniques coupled with fluorescent biomarkers. Strategies for labelling GBS with fluorescent biomarkers, have so far been limited to antibody-based immunostaining methods and nonspecific protein/DNA stains.

Green fluorescent protein (GFP) expression is a common labeling method for the study of bacteria. It enables the identification of bacteria in complex samples, the monitoring of subcellular locations of bacteria in eukaryotic host cells, and the development of novel and efficient enumeration methods such as based on flow cytometry.

In a recent study¹ we have reported stable expression of a green fluorescent protein mutant (GFPmut3) in GBS. This was achieved through the use of GFP-variant-expressing plasmid, pGU2664. GFPmut3 has 20x greater fluorescence intensity than GFP, allowing greater sensitivity for visualization of GBS². Herein, we describe absorbance, fluorescence and fluorescence polarisation intensity experiments aimed at validating and monitoring the stability of GFPmut3 expression in GBS cultures. A detailed account of microscopic analysis, effect of antimicrobials on GFPmut3 expression in GBS and adhesion studies of GFP-expressing GBS to human epithelial cells is described in *Applied and Environmental Microbiology*¹.

Assay Principle

Polarised fluorescence intensity

Fluorescence polarisation measures rotation of molecules in solution. High molecular weight species (like GFP) rotate slowly and when excited with plane polarised light emit a high proportion of polarised light back to the detector. Low molecular weight compounds rotate quickly and re-emit unpolarised light. The degree of polarisation

(P), is defined as $(I_{para} - I_{perp}) / (I_{para} + I_{perp})$, where I_{para} is the fluorescence intensity measured parallel and I_{perp} is that measured perpendicular to the plane of polarization of the excitation light.

For slow rotating species in solution the Polarisation value can be as high as 0.4 (400mP). For low molecular weight, quickly rotating molecules this value is very low (35mP or less) because $I_{para} - I_{perp}$ gives a very small value in the above equation due to the intensities being very nearly the same. In our study, we have made use of this property to monitor GFPmut3 fluorescence over background autofluorescence in the commonly used bacterial medium for growth of GBS, Todd-Hewitt Broth (THB). Polarisation intensities, I_{para} and I_{perp} , were measured temporally over a 12-hour period and total fluorescence polarisation intensity was calculated by the sum $I_{para} + 2I_{perp}$.

Material & Methods

- Microplates (96 well, Greiner Cat No. 655185)
- CLARIOstar with atmospheric control unit (ACU)
- Plates were sealed using gas-permeable BreatheEasy membranes [Sigma, Cat No. Z380059]

Experimental Procedure

Overnight THB cultures of GBS str 874391 were harvested by centrifugation (8,000 x g, 10 min) and washed three times with PBS (pH 7.4). Bacteria were subsequently diluted 1:100 into fresh Chemically Defined Medium (CDM) or THB (approx. 1×10^6 CFU per well) and 200 ul volumes of the cell suspensions were aliquoted in triplicate into wells of a 96 well plate.

Plates were sealed with breathe-easy membrane and incubated for 12h in the CLARIOstar multi-mode platereader, with temperature set to 37 °C and a 5 % CO₂ atmosphere.

Absorbance at 600nm [OD₆₀₀], Fluorescence Intensity and/or Polarised fluorescence intensity was simultaneously monitored every 15 min with agitation at 300 rpm between cycles.



Instrument Settings

Optic Settings	Absorbance	
	Discrete wavelength	600 nm
	Pathlength -correction	200 μ l
	Fluorescence Intensity	
	LVF	470-15 Auto 488.8 515-20
	Gain	1700
	Fluorescence Polarization (FP)	
	Filters	Ex 482-16 LP504 EM 515-30
	Target mP	35
	Gains	A: 951; B 1113
General settings	Number of flashes	10, 50 for FP
	Settling time	0.2 s
	Orbital averaging	3 mm
Kinetic settings	Number of cycles	74
	Cycle time	900 s
Incubation	37 $^{\circ}$ C	
Atmospheric Control	CO ₂ : 5%; O ₂ : monitoring	

Results & Discussion

The GFPmut3 plasmid pGU2664 was introduced, via electroporation, into GBS 874391 to create GBS strain GU2666 (GFP+). In addition, a non-GFPmut3 vector was introduced to create strain GU2672 (GFP-).¹

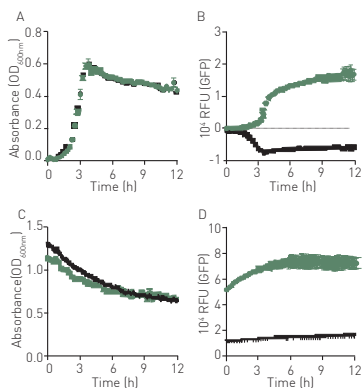


Fig. 1: Absorbance (A) and Fluorescence intensity profile (B) of GFP+ GBS (green) and GFP- GBS (black) grown in CDM media, or Absorbance (C) and Fluorescence profiles (D) of stationary phase cells in a standard buffer (PBS).

To assess the burden of GFPmut3 expression on the physiology of GBS, we measured the growth of GBS by simultaneously monitoring cell density (optical density at 600nm) and GFPmut3 fluorescence intensity in CDM. The growth rates of GFP+ bacteria were identical to

GBS containing the empty vector (GFP-), suggesting no significant metabolic burden to the GBS strain carrying the pGU2664 plasmid (Fig. 1A). In addition, there was strong GFP emission that correlated well with cell density, demonstrating expression and stability of GFPmut3 across exponential and stationary growth phases (Fig. 1B).

The fluorescence of GFPmut3 was also examined under stationary phase and low nutrient conditions. Cells were grown overnight, re-suspended in PBS and monitored for turbidity and fluorescence over 12h, as described above. We observed stable and high-level fluorescence for these stationary phase populations with turbidity remaining stable (Fig 1, C & D).

THB is a standard complete media commonly used for growing GBS for infection experiments in animal or cell culture-based methods. We therefore also investigated the expression of GFPmut3 fluorescence during growth in THB. Interestingly, the THB media contains compounds that autofluoresce at 515nm, which interfere with the GFP signal (Fig 2B). This could be conveniently overcome by monitoring GBS growth via polarised fluorescence intensity over the 12 hour period (Fig 2C).

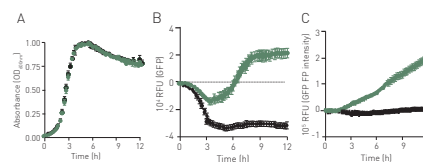


Fig. 2: Absorbance (A), Fluorescence (B) and Polarised Fluorescence (C) profiles for GBS (GFP+, green; GFP-, black) grown in THB.

Conclusion

Taken together, the above results demonstrate a new method for analyzing GFPmut3 expressing GBS in experimental systems, emphasising the usefulness of the FP intensity monitoring technique in microbiology. This study also highlights the versatility of the CLARIOstar multimode platereader with ACU for microbiology. The instrument can incubate and shake samples under a controlled atmosphere (CO₂ and/or O₂) while acquiring data using various techniques (FL, Lum, Abs, FP) simultaneously. In our laboratory, this has become an essential tool for investigating bacterial survival and the mechanisms of infection.

References

- Sullivan, MJ, Ulett, GC. Applied Environmental Microbiology. [2018] 84: 1-18. DOI: 10.1128/AEM.01262-18.
- Cormack BP *et al.* Gene [1996] 173: 33-38. DOI: 10.1016/0378-1119(95)00685-0 *Copyright © American Society for Microbiology, Applied and Environmental Microbiology, 84 (18), 2018, e01262-18

