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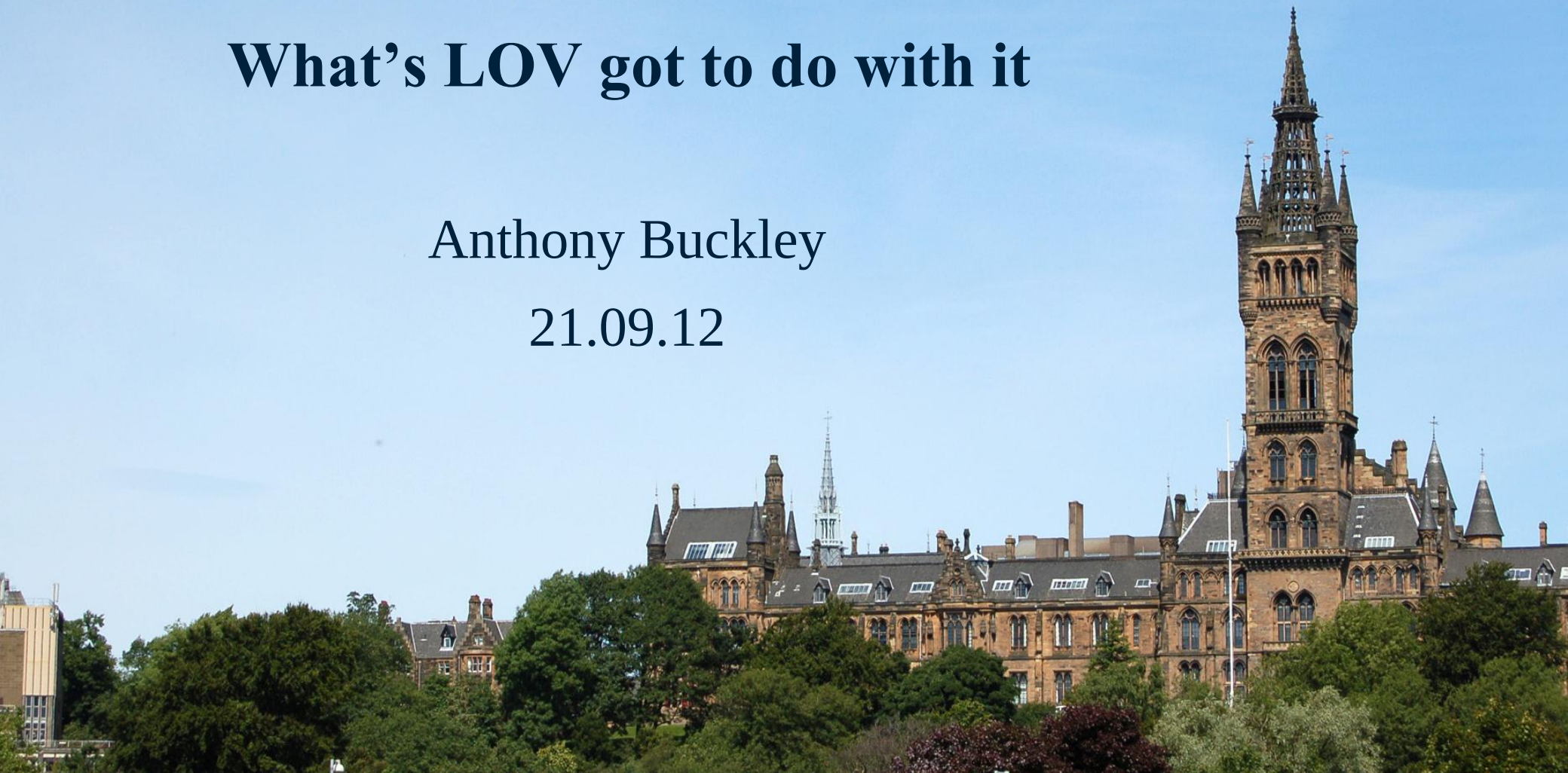
What's LOV got to do with it

Anthony Buckley

21.09.12



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Visualising *C. difficile*

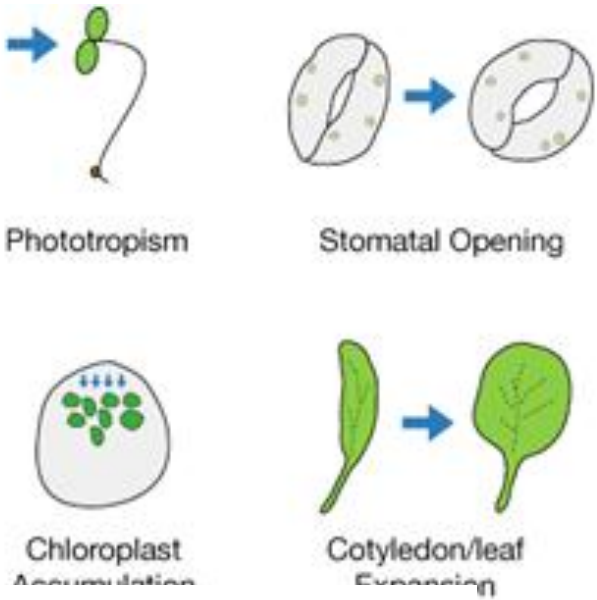
- Current methods to visualise *C. difficile* using fluorescent reporters, such as GFP, have been hampered by their requirement for oxygen.
    - *C. difficile*-specific antibody
        - anti-cwpV / anti-pilA / flagella
    - Fluorescent *in situ* hybridisation
        - 16S rRNA specific probe

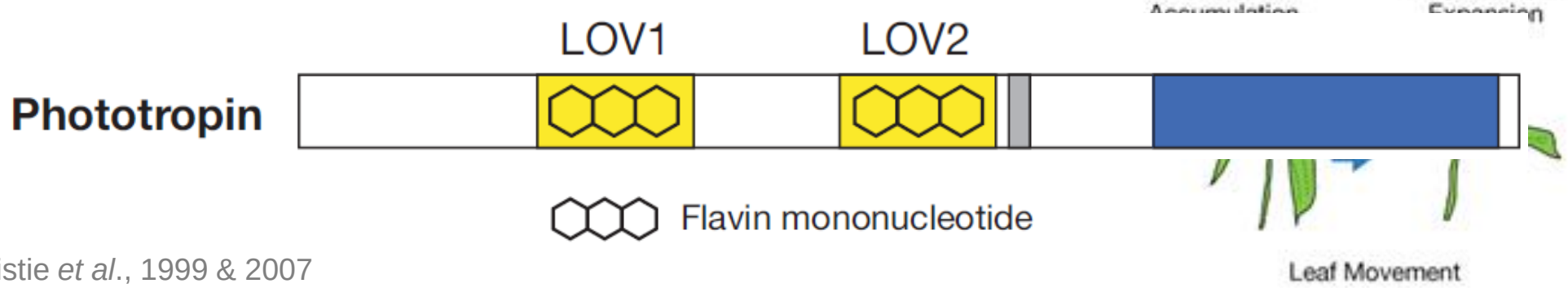
***Use of LOV domains as a fluorescent reporter in* C. difficile**

Milinovich *et al*., 2007 & Reynolds *et al*., 2011

A leaf out of a botanist's book

- Phototropins are blue-light receptors control a range of responses that optimise photosynthetic efficiency of plants.
- Plasma membrane-associated Ser/Thr photoreceptor kinases that undergo autophosphorylation.
- N-terminal photosensory input & C-terminal effector domain.





Christie *et al*., 1999 & 2007

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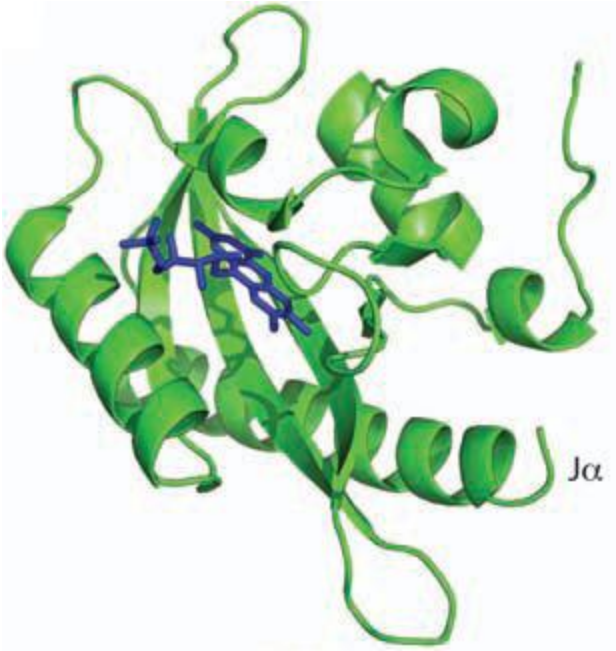
What is LOV?

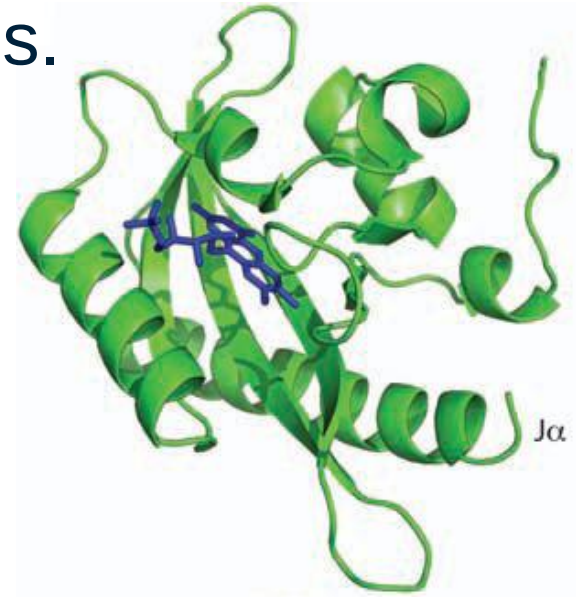
- Light, Oxygen or Voltage sensing domain (LOV).
- Function as versatile photosensory modules.
- Bind flavin mononucleotide (FMN) as a co-factor.
- Consists of 5 anti-parallel β-sheets & 2 α-helices.
- LOV-containing proteins found in plants, fungi & bacteria.

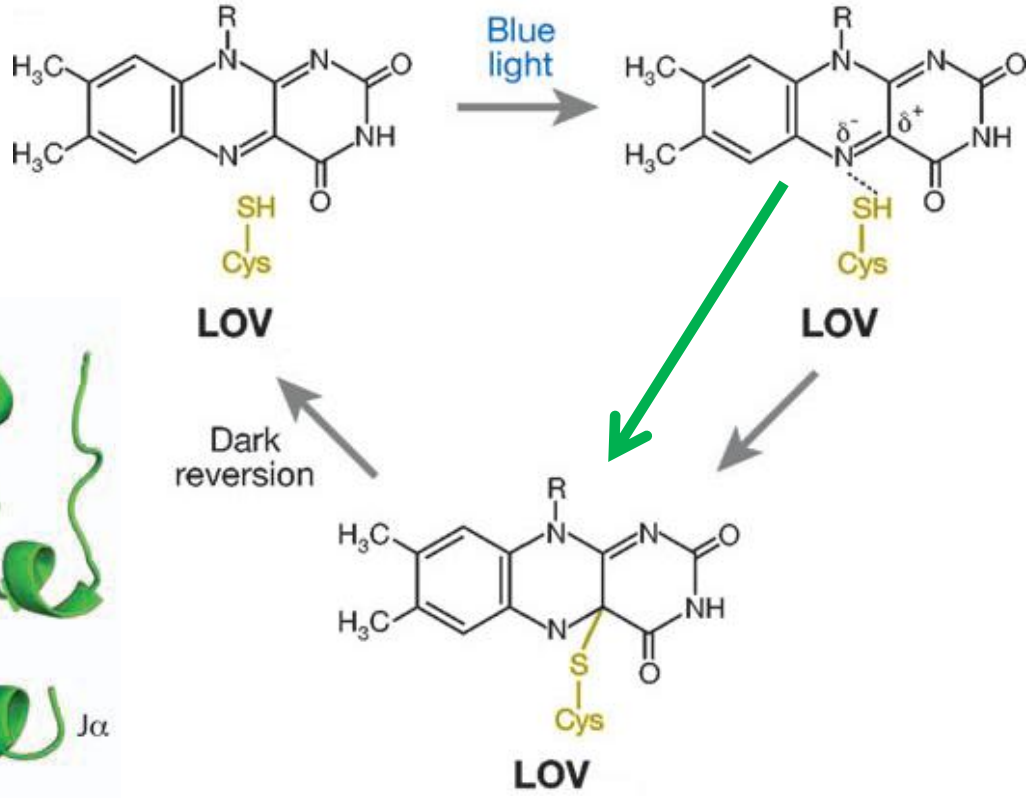


Christie *et al*., 2007 & 2012

The photoreversible reaction

- In darkness, LOV binds FMN noncovalently.
- Upon blue light irradiation LOV_{C426} covalently binds FMN.
- Activation of the C-terminal kinase domain.
- LOV domains can act as reporter proteins.



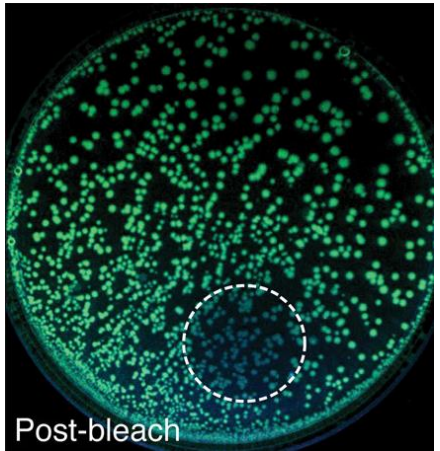


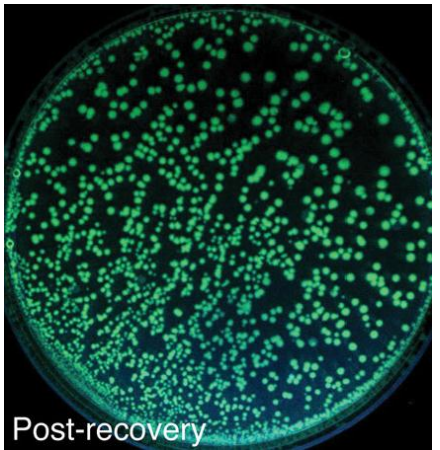
Christie *et al*., 2007 & 2012

Advantages of LOV

- Several advantages over GFP-related fluorescent proteins

1. Small size (~11kDa), less toxic & soluble
2. Fluorescence stable over a greater pH range
3. Reversible photobleaching
4. Fluorescence is independent of molecular oxygen

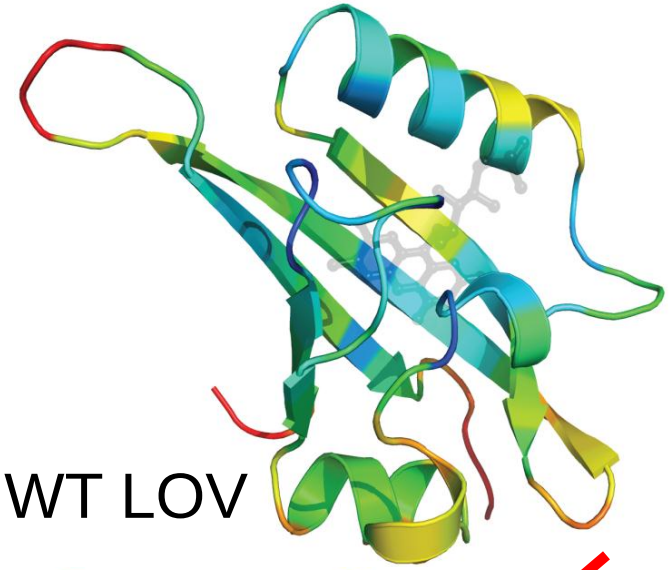




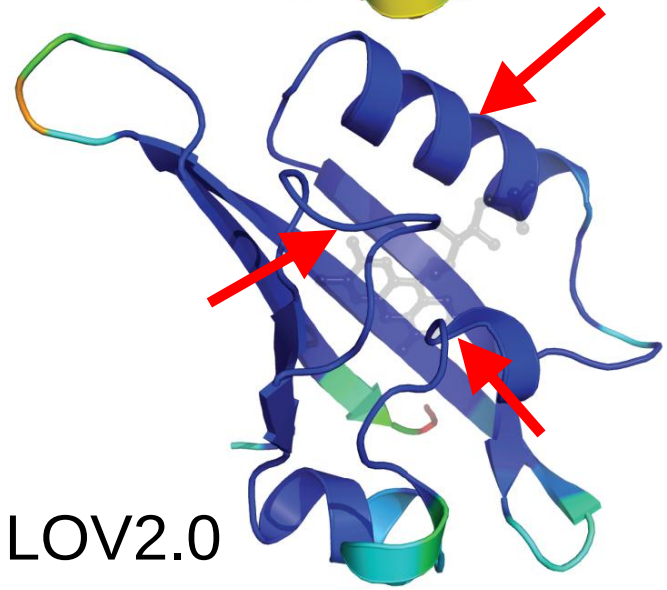
Christie *et al*., 2007 & 2012

Improving LOV structure

- LOV domain from *Arabidopsis thaliana*.
- Directed evolution used to increase fluorescence & photostability, resulting in LOV 2.0.
- Codon optimisation 27% of nucleotides, mostly C to A, 100% amino acid identity.



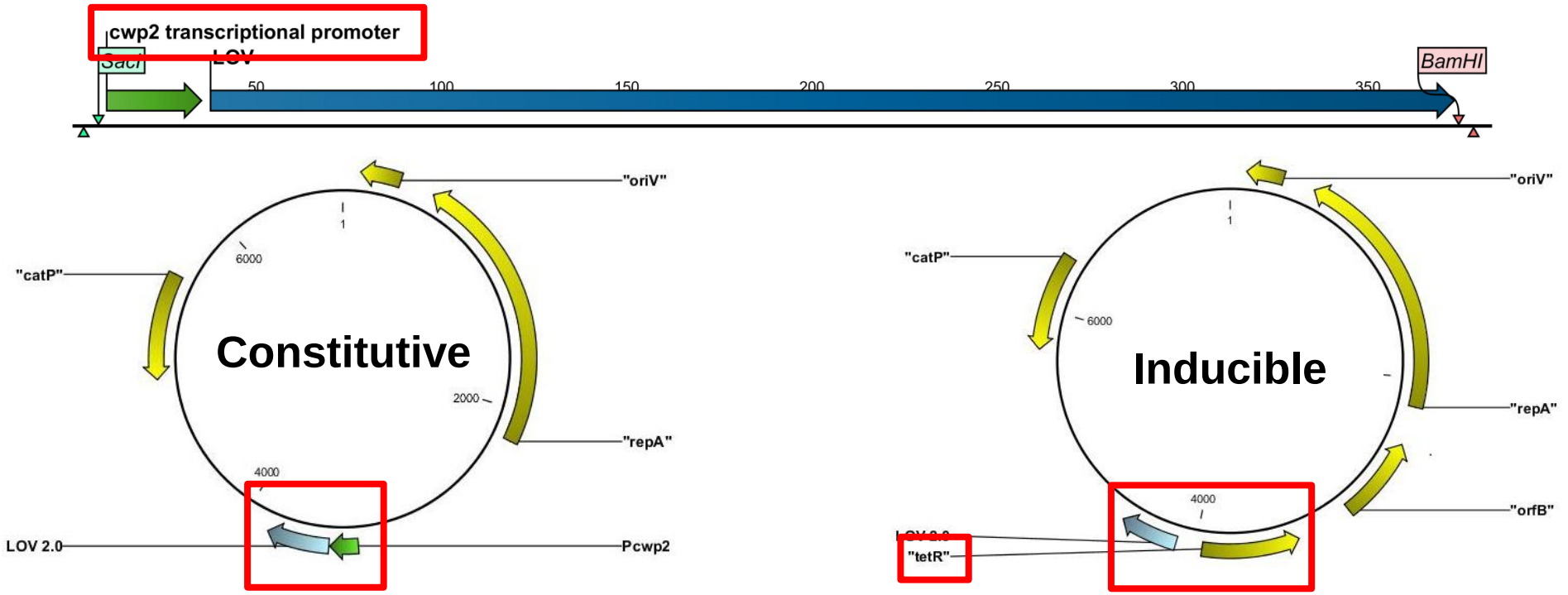
WT LOV



LOV2.0

Chapman *et al*., 2008 & Christie *et al*., 2012

Express your LOV

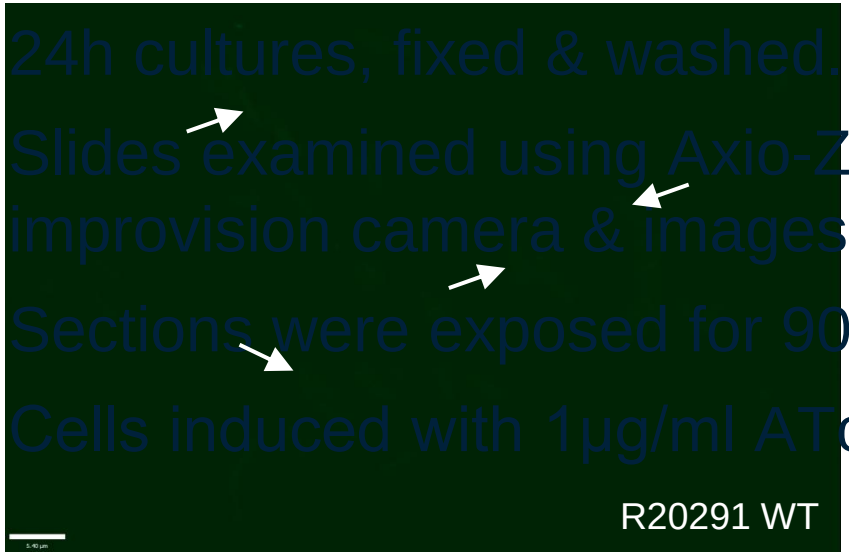


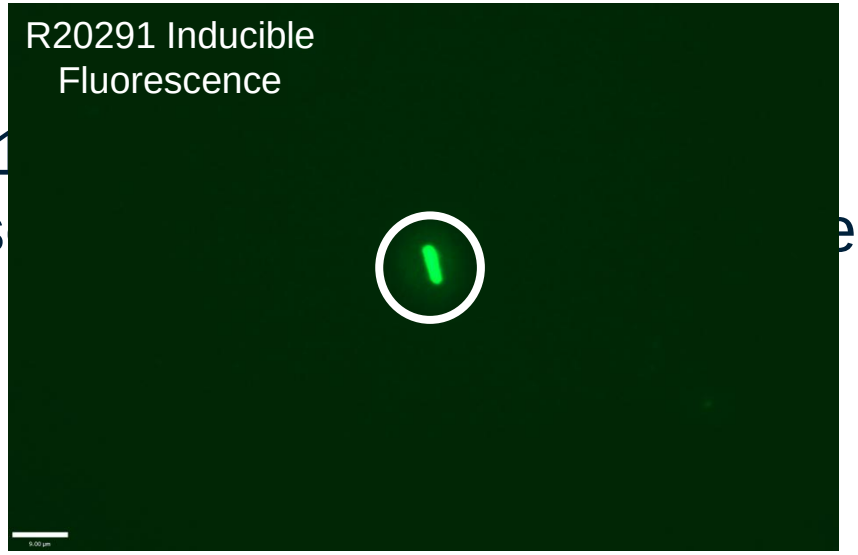
- Conjugated *C. difficile* R20291
- Ribotype 027 & toxinotype III
- Isolated during 2004 U.K. outbreak
- Fully virulent in the hamster model of infection

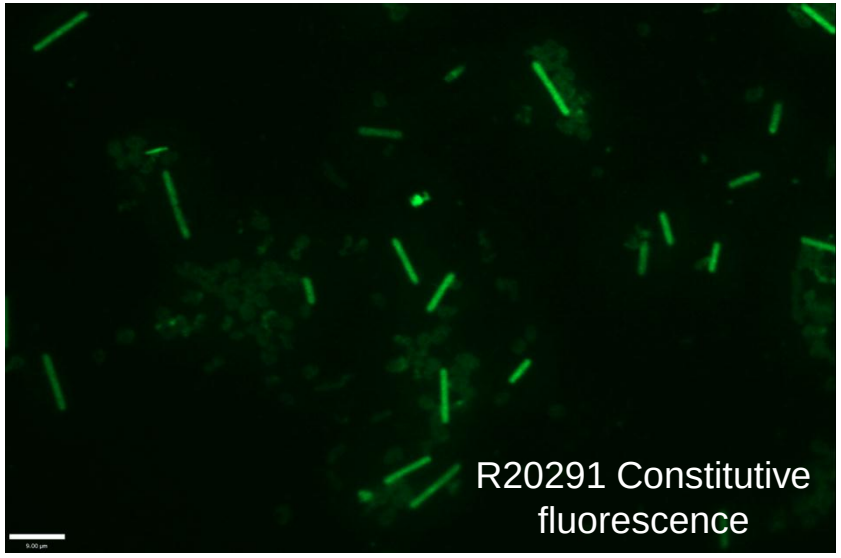
Buckley *et al*., 2011
& Fagan *et al*., 2011

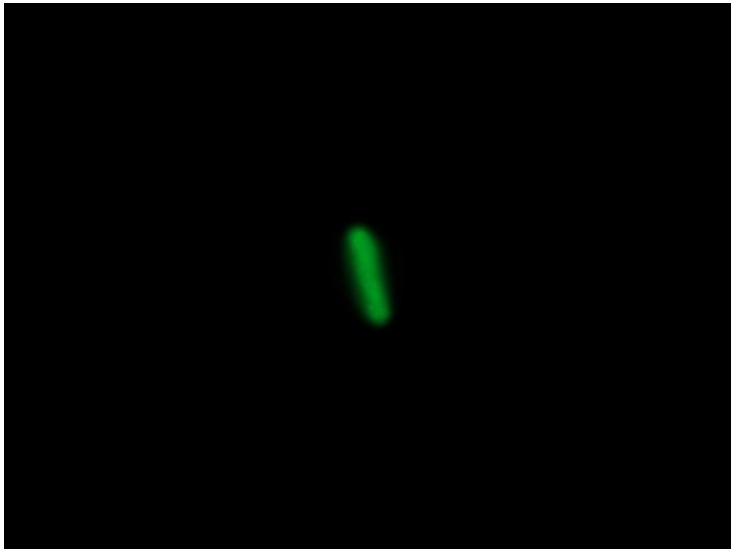
The LOV bug

- 24h cultures, fixed & washed.
- Slides examined using Axio-Zeiss M1 improvision camera & images analys e.
- Sections were exposed for 900ms.
- Cells induced with 1µg/ml ATc for 1h



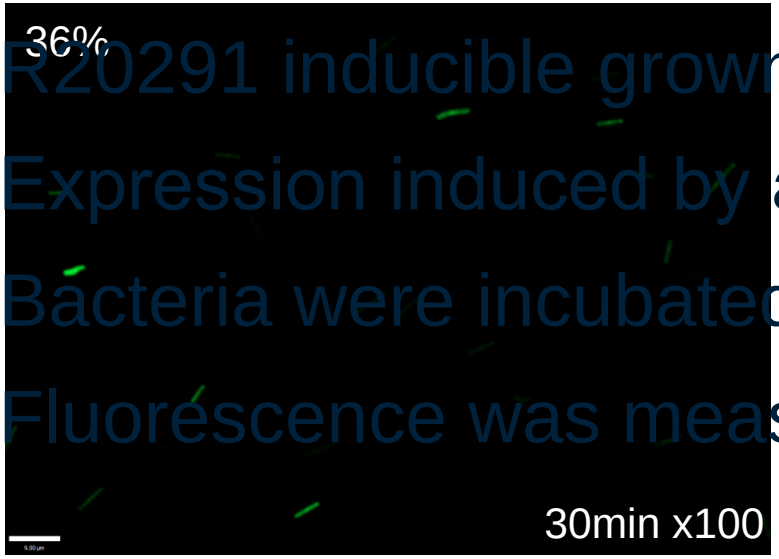


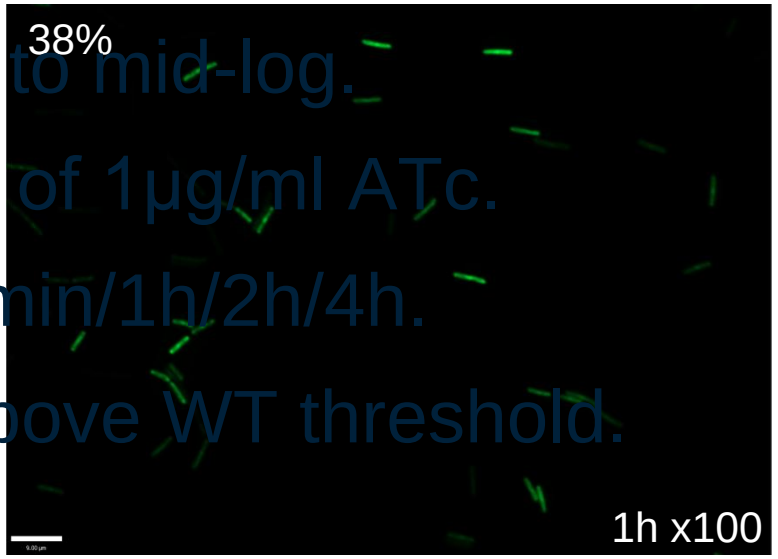


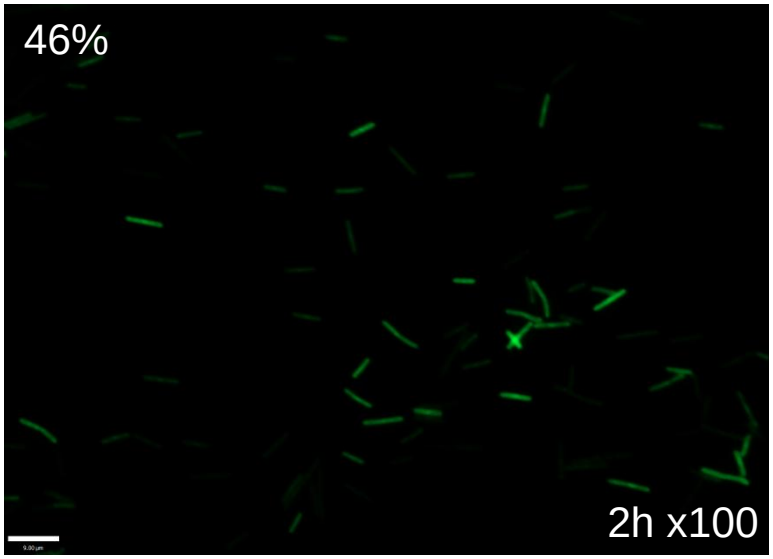


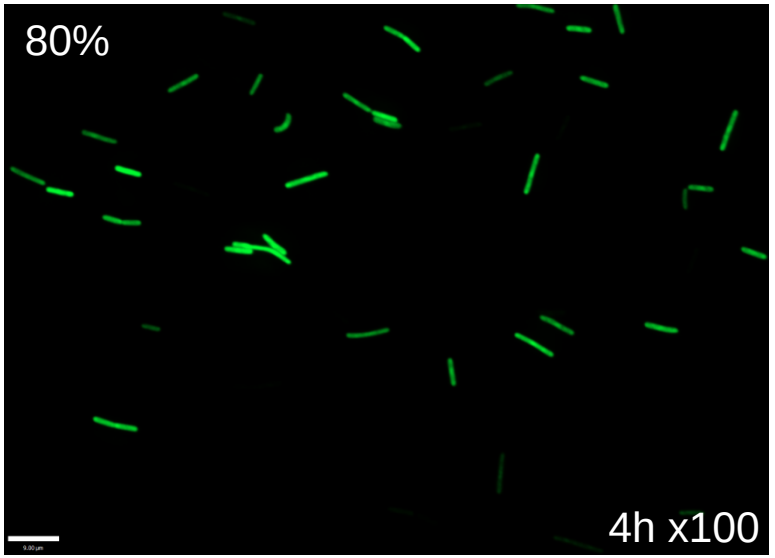
Induction of LOV

- R20291 inducible grown in BHI to mid-log.
- Expression induced by addition of 1µg/ml ATc.
- Bacteria were incubated for 30min/1h/2h/4h.
- Fluorescence was measured above WT threshold.



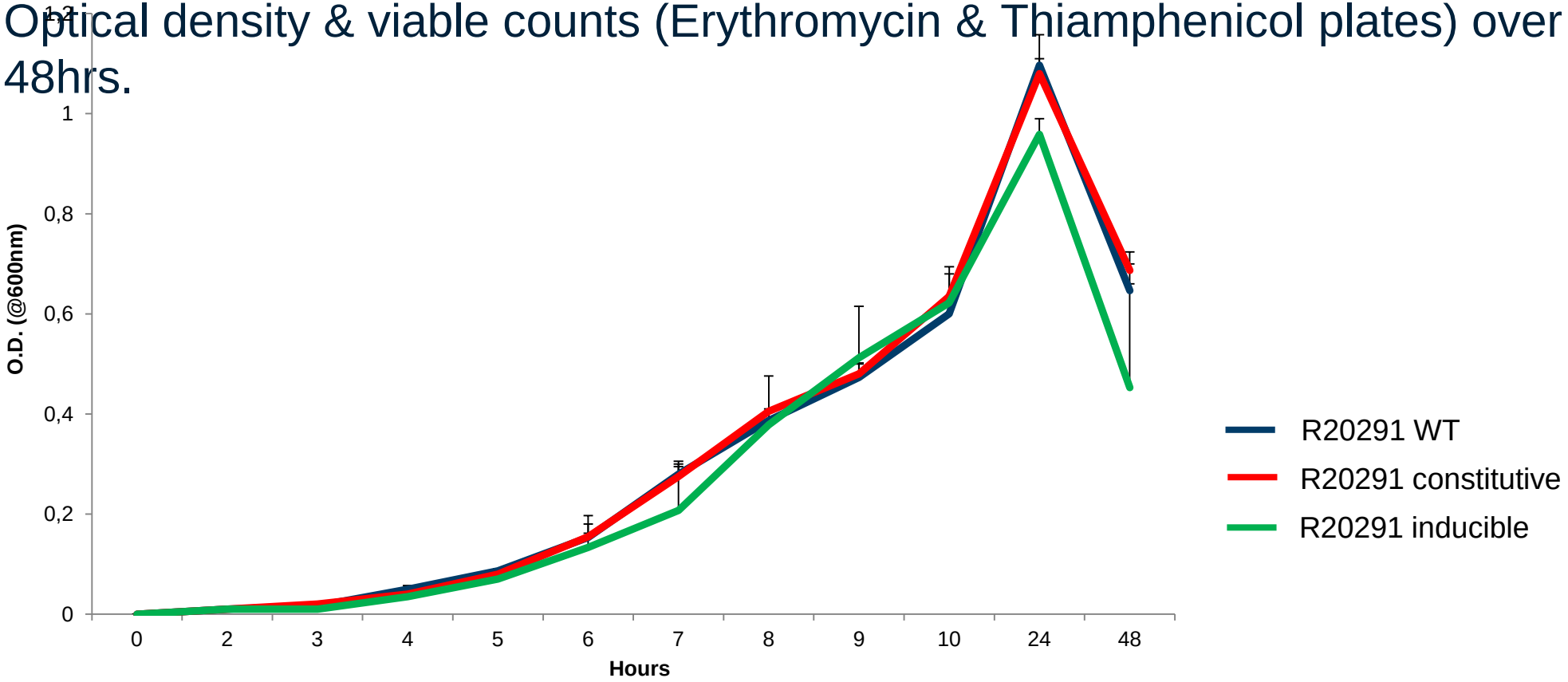






in vitro growth assay

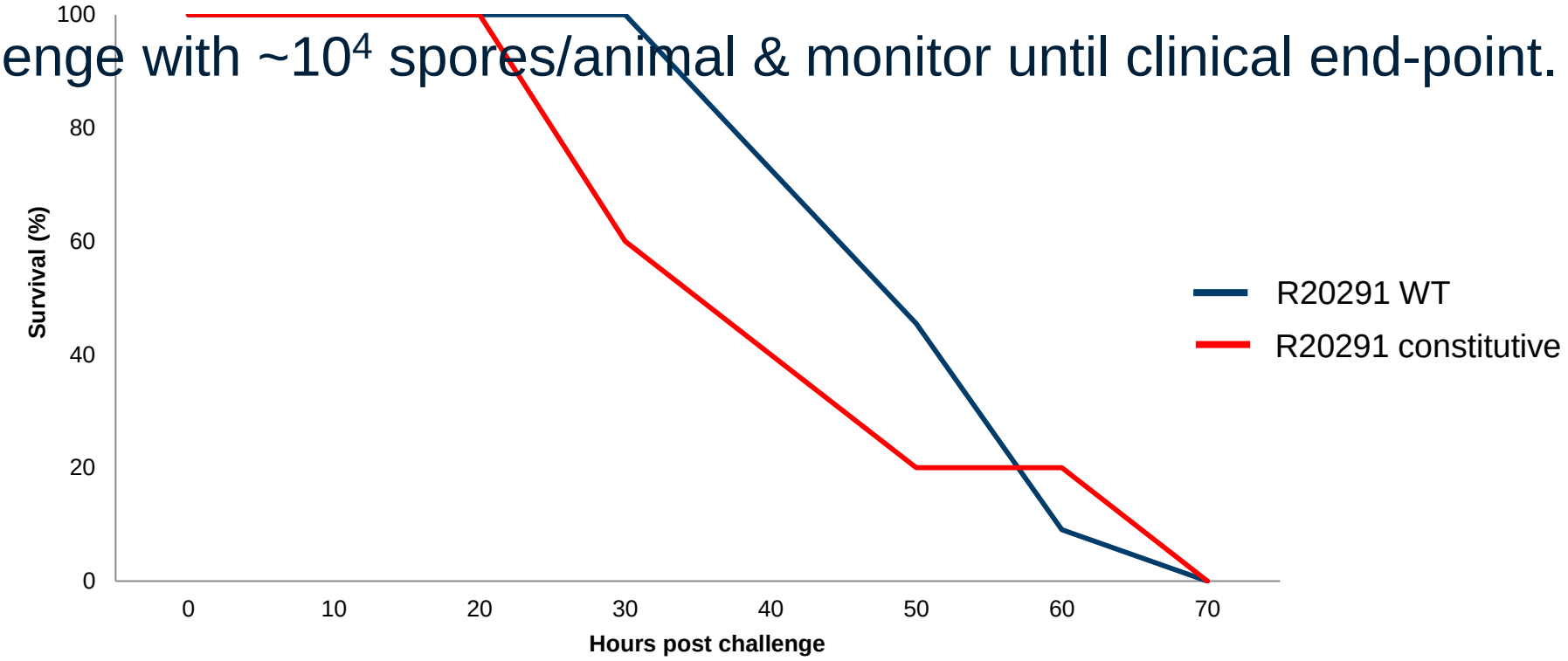
- Strains grown in BHI broth.
- Optical density & viable counts (Erythromycin & Thiamphenicol plates) over 48hrs.



- Average plasmid stability for R20291 constitutive & inducible is 88% & 80%, respectively.

Hamster challenge

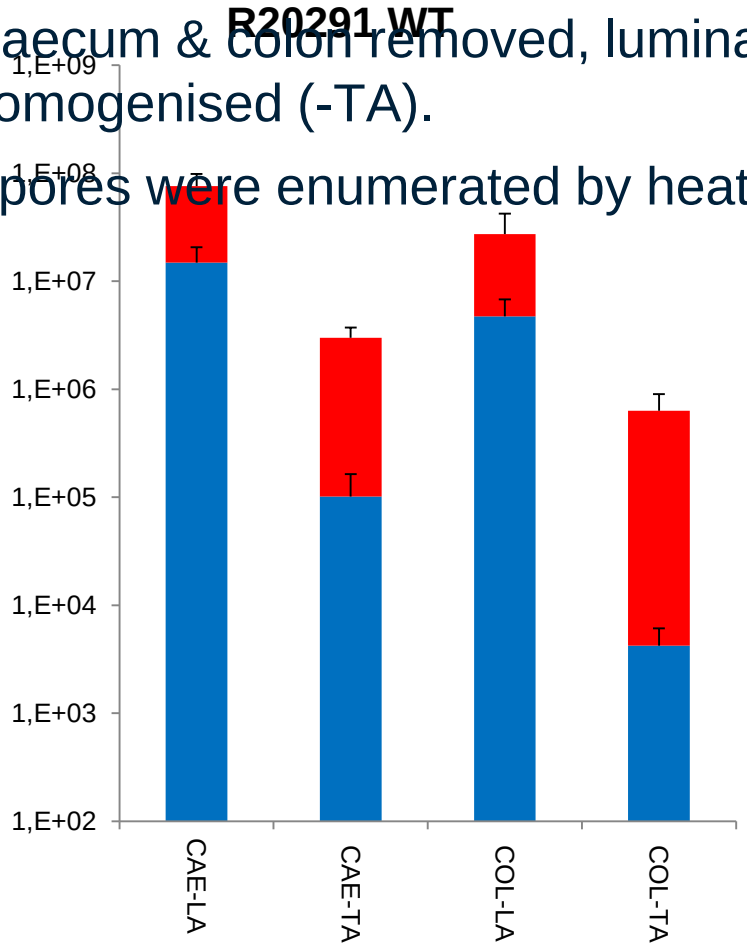
- Using a 5-day clindamycin treatment.
- Challenge with ~10^4 spores/animal & monitor until clinical end-point.

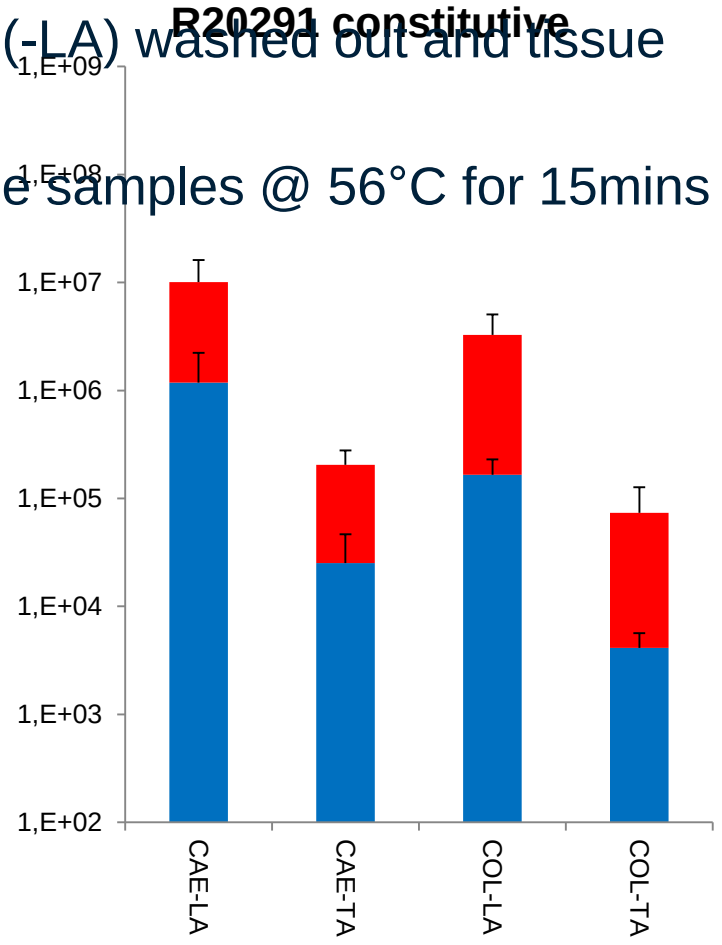


Strain	Expression	Number	Mean time to cull	Range	*p* value
R20291 WT		11	46h 43m ± 2h 52m	36-62h	
R20291 p144-LOV	Constitutive	5	43h 6m ± 6h 5m	31-65h	0.54

in vivo colonisation

- Animals culled at clinical end-point.
- Caecum & colon removed, luminal contents (-LA) washed out and tissue homogenised (-TA).
- Spores were enumerated by heat-treating the samples @ 56°C for 15mins

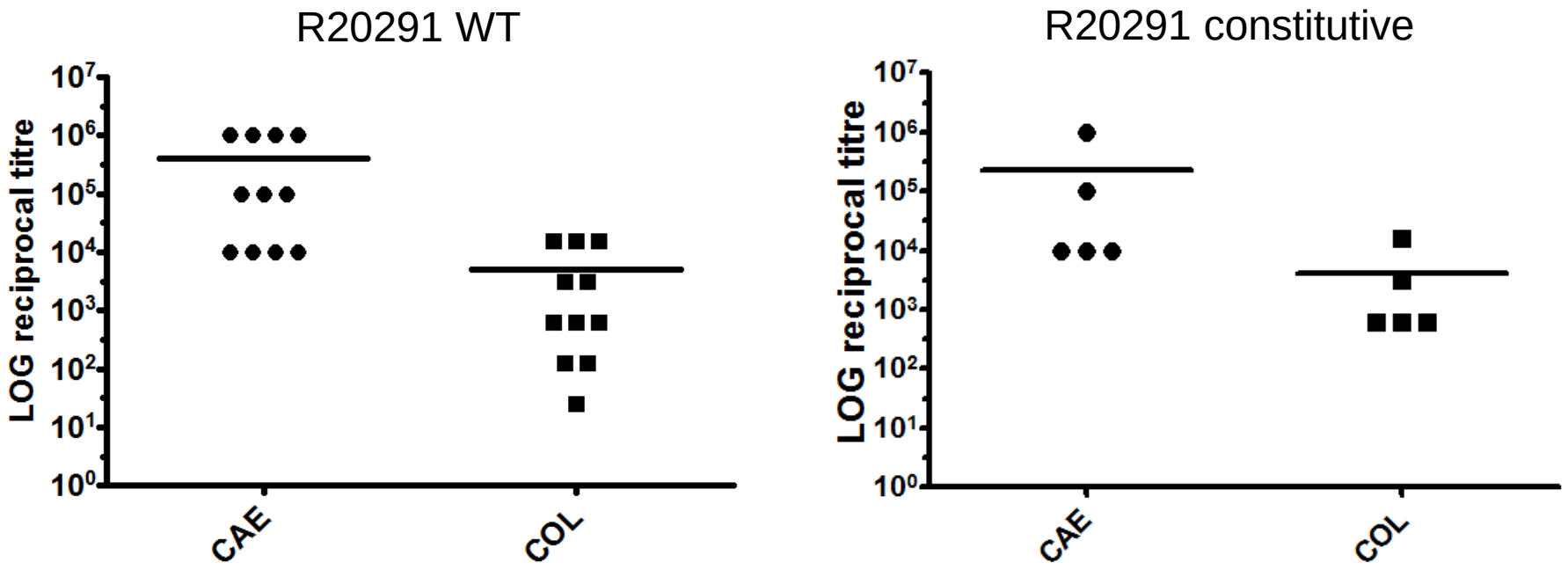




- Average plasmid stability 84% across all organs

in vivo toxin assay

- Serial dilutions of gut contents incubated with Vero cells overnight.
- Cells washed, fixed and stained with Giemsa.



- Mean toxin measurements show no significant differences between strains.

Tissue microscopy

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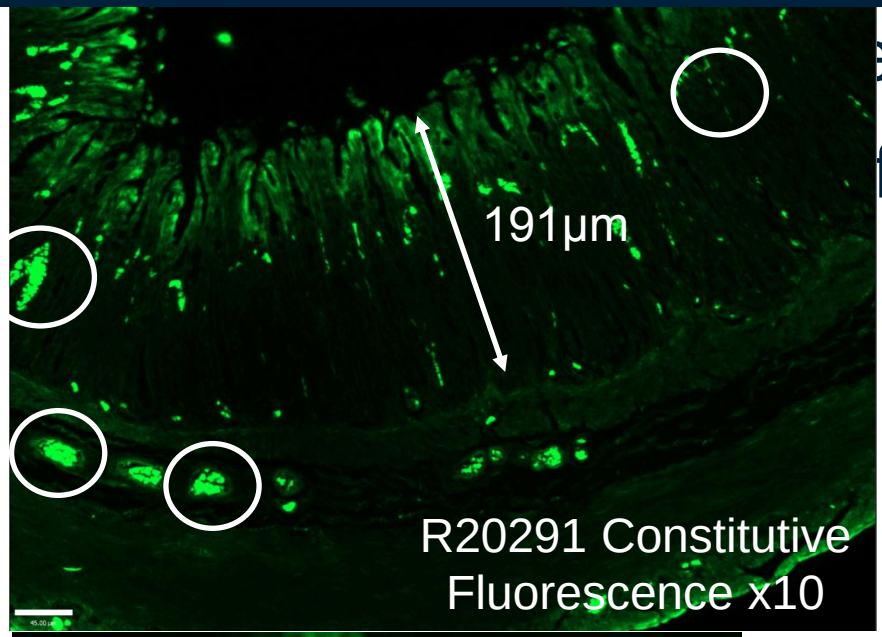
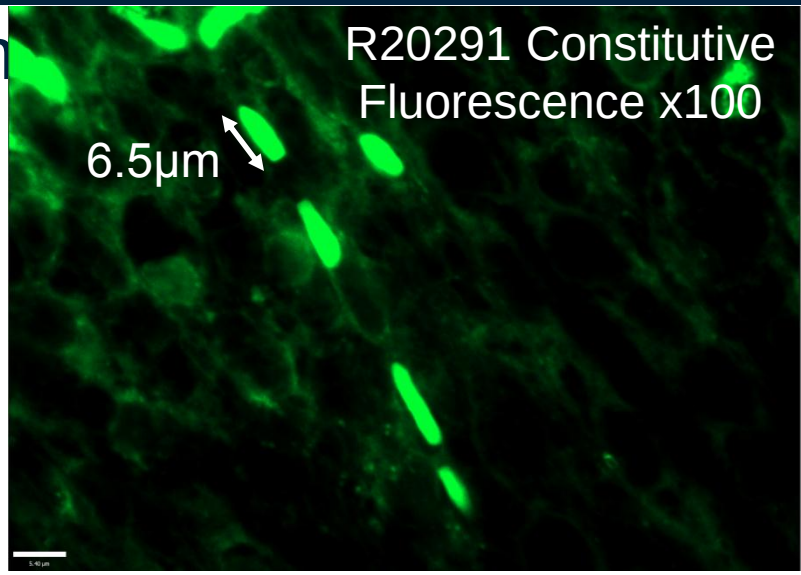
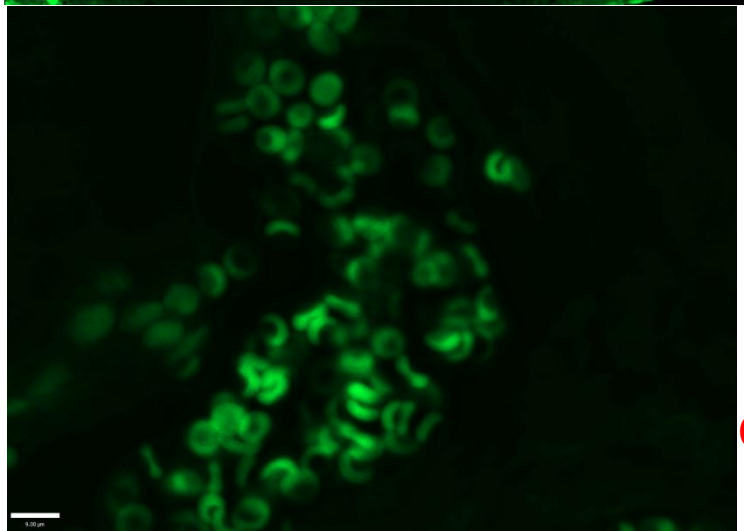
for 200ms.

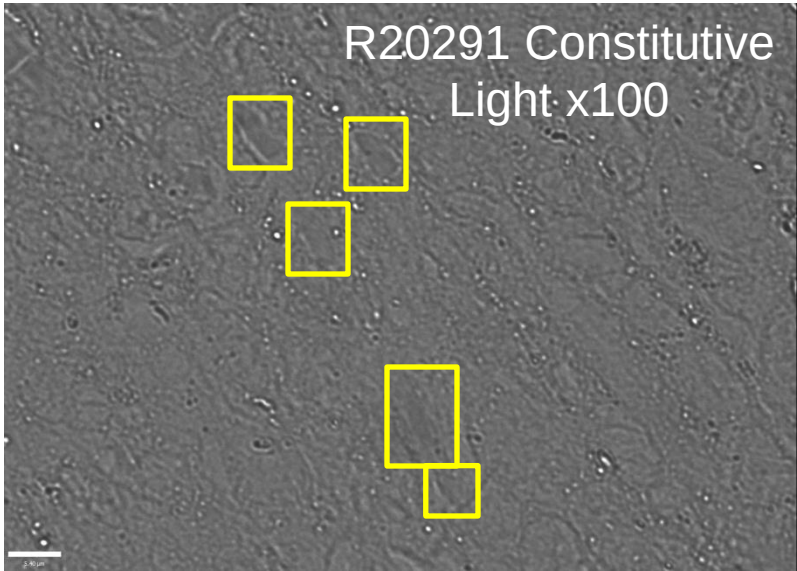






Caution! red blood cells

Fluorescence



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Discussion

- Directed evolution of LOV domains increase fluorescence & photostability.
- Temperature stable variants (60ºC)
- LOV domains can act as fluorescent reporter in anaerobic systems, such as *C. difficile*.
- LOV causes *C. difficile* to fluoresce green
- Show no significant *in vitro* growth differences
- Have similar *in vivo* characteristics
- LOV has the potential to be used to determine tissue localisation of *C. difficile* strains
- Highlights the use of LOV domains in the emerging field of optogentics

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