High pressure induced DNA damage in *Escherichia coli* involves pressure-mediated dissociation of the tetrameric Mrr restriction endonuclease

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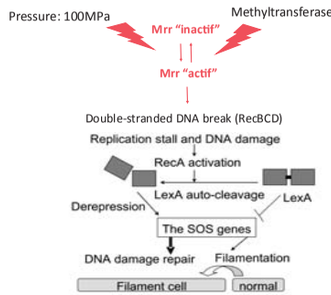
Abstract: Pressure shock of ~100 MPa elicits a *bona fide* DNA damage response in the mesophilic *E. coli* bacterium, despite the fact that pressure, itself, cannot compromise DNA integrity. While *E. coli* is not naturally subjected to high hydrostatic pressure (HP) in its environment, food products are often pressure treated to inactivate food-borne pathogens. Screens for resistance to pressure shock by Aertsen's group revealed an endogenous restriction endonuclease, Mrr (Methylated adenine Recognition and Restriction), that cleaves methylated DNA [1,2]. Since expression of Mrr is not toxic for the cell in the absence of HP, pressure stress must somehow be able to elicit Mrr activity. We used fluorescence fluctuation microscopy of strains chromosomally expressing a GFPmut2-Mrr fusion to investigate HP activation of Mrr. In scanning mode, FFM provides a spatial map of the absolute concentration (=n/PSF) and stoichiometry (=brightness, e) of the fluorescent particles [3]. Before HP shock GFPmut2-Mrr exhibits a stoichiometry consistent with a tetramer. It appears to be slightly preferentially localized around the chromosome, diffusing more slowly than free GFP, yet not being immobile. After 20 min at 100 MPa followed by pressure release, GFPmut2-Mrr is found accumulated in foci that co-localize strongly with the chromosome, as previously observed [4]. Interestingly, the mobility of the Mrr in these foci is significantly decreased and their stoichiometry is consistent with a dimer. The Mrr molecules which are excluded from the foci remain tetrameric. We suggest that pressure-mediated triggering of Mrr activity and the concomitant generation of DNA damage involves the conversion of inactive Mrr tetramers to active Mrr dimers. This could occur either via a direct effect of pressure on the Mrr tetramer-dimer equilibrium, a common effect of pressure on oligomers, or via a pressure effect on chromosome structural that leads to more efficient recruitment of active dimeric Mrr.

Subjecting E. coli cells to pressure shock of ~1 kbar induces the SOS response, and double-stranded DNA breaks are observed in the host chromosome. While the response after the double stranded DNA breaks uses the same factors as UV induction and is recA, recB and lexA dependent. Considering that 100MPa shock cannot lead to cleavage of dsDNA, another factor upstream of SSB and RecA must be involved in the response to pressure shock. Although *E. coli* is not naturally subjected to high pressure (HP) environments, food are often subjected to a combination of bacterial stresses, such as HP, to inactivate food-borne pathogens such as *Salmonella* and *E. coli* O157:H7. The Aertsen group (KU Leuven, Belgium) identified an endogenous type IV restriction endonuclease with no cognate methyltransferase Mrr (Methylated adenine Recognition and Restriction) that is required for the SOS response to pressure shock and that specially targets methylated DNA. Mrr is inactive at ambient pressure and is activated upon pressure shock. We have set out to determine the molecular mechanism of pressure-activation of Mrr using a novel quantitative microscopy approach called N&B.

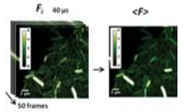


SOS response activation by high pressure or a methyltransferase (HhaII) via Mrr.

There are two way to activate Mrr and elicit the SOS response: High pressure shock and expression of the methyltransferase (HhaII) that is not present in *E.coli* [5].

In vivo experiments: Number and Brightness (N&B)

This technique is based on a moment-analysis of the fluctuations in fluorescence of a tagged protein diffusing in live cells. Using the fluctuations in fluorescence in a small two-photon excitation volume scanned over the Field of View (FOV), the average number of molecules (n) and brightness (e) of each pixel in fluorescence images can be calculated, providing a spatial map of protein concentration and stoichiometry.



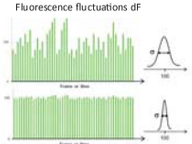
Number of fluorescent particles = concentration

Fluorescence intensity — Brightness of fluorescent particles = stoichiometry

$$F = n \times e$$

$$n = \frac{<F(t)>^2}{<\delta F(t)^2> - <F(t)>} \qquad e = \frac{<\delta F(t)^2> - <F(t)>}{<F(t)>}$$

Fluorescence fluctuations dF



<F> = 100 = 10 x 10

<F> = 100 = 100 x 1

Actually, normal fluorescence microscopy techniques measuring only the fluorescence intensity can be misleading because few bright particles or many less bright particles are indistiguishable. But with the fluorescence fluctuations at each pixel in a stack of 50 images, n and e can be calculated from the variance and the average intensity.

The e and n values are corrected for shot noise, auto-fluorescent background and photo-bleaching.

$$\text{Stoichiometry} = \frac{e_{\text{cells}}}{e_{\text{gfpmut2 monomeric}}}$$

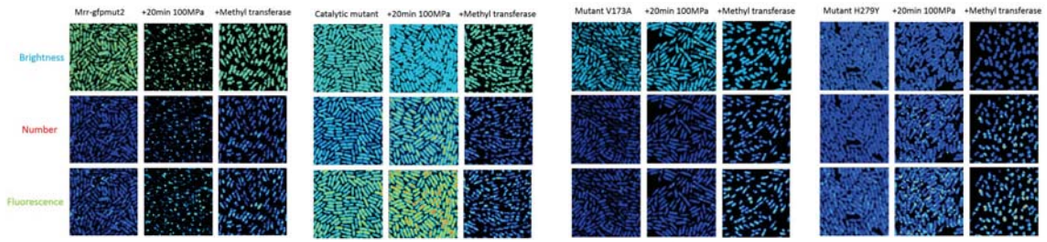
Direct *in vivo* measurement by measuring brightness of a fluorescent proteins fused with our proteins of interest:

-> protein oligomerization

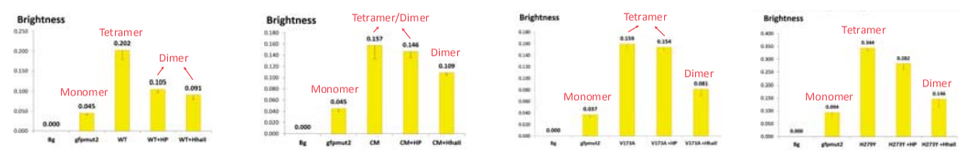
-> protein concentration

-> protein localization

In vivo results



***E. coli* strain MG1655 harboring a chromosomal Pbad-gfp-WT Mrr or its catalytic mutants D203A, H273Y and V173A were pressurized for 20min at 100MPa or induced with the methyltransferase HhaII. Computer-controlled HUB440 high pressure generator equipped with the SW-16 pressure vessel (Pressure BioSciences, Inc., South Easton, MA) was used to pressurize samples in 50uL MicroTubes (also from Pressure BioSciences, Inc.). Then pressure was released. Mapping of e, n and f was carried out by imaging the cells incubated with or without pressure. Brightness values are shown after correction for photo-bleaching, shot noise and background contribution (analyzed with SimFCS and Patrack).**



Brightness values corrected by the background fluorescence. Cells were growth in LB and induced with 0.4% arabinose for 3h before HP treatment or HhaII induction 1mM IPTG for 1h.

N&B conclusions for WT Mrr-gfpmut2:

- **Before High pressure Mrr is a tetramer.**
- **After HP stress, Mrr tetramers dissociate to dimers that interact with DNA in foci.**
- **Upon activation by HhaII, Mrr also shifts to a dimer and hyperfilamentation of cells is observed, indicative of a strong SOS response.**
- **The methyl transferase also active Mrr. Focis appears and the brightness inside reveal dimers.**

Catalytic mutant D203A:

- **Mutation impacts the Mrr-gfpmut2 tetramer-dimer equilibrium at atmospheric pressure.**
- **No apparent effect of pressure; brightness is the same before and after pressure shock.**
- **Same effect of the methyltransferase**

Mutant V173A:

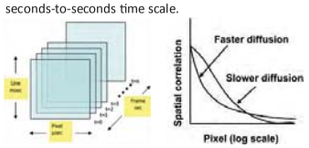
- **Mrr is a tetramer before HP shock.**
- **No apparent effect of pressure; brightness is the same before and after.**
- **Same effect of the methyltransferase as the WT.**

Mutant H279Y:

- **Tetramers before HP shock.**
- **No clear effect of HP shock, brightness is slightly lower.**
- **Mrr is dimeric but there is no hyperfilamentation of cells. Mrr might bind DNA but doesn't cut it.**

In vivo experiments: Raster Imaging Correlation Spectroscopy (RICS)

The same data as N&B are used to calculate diffusion coefficient (D) and detect interaction by measuring changes in diffusion coefficients. RICS is temporally resolve from micro-seconds-to-seconds time scale.



Molecules moving on the timescales of the scan speed which is tens of microseconds along horizontal lines and milliseconds along vertical lines.

Spatial correlation representation of diffusing particles. Correlation increase with a slow diffusion.

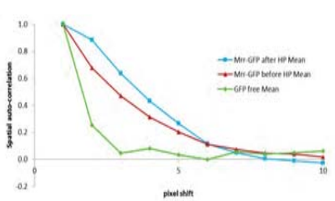
These autocorrelation function allows the measurement of:

-> protein diffusion

-> protein interactions

Results with Mrr-gfpmut2 WT:

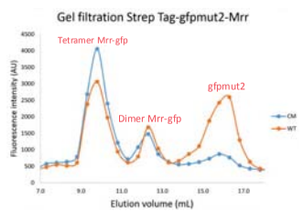
Autocorrelation representation of free Gfpmut2 diffusing in cells and Mrr-gfpmut2 before and after an HP shock (20min 100MPa).



RICS conclusion:

- **Mrr-gfp diffusion is slower after an HP shock due to a close interaction between Mrr-gfp dimers and DNA.**
- **A higher proportion of immobile fluorescent Mrr is observed after pressure. This likely corresponds to Mrr bound to DNA.**

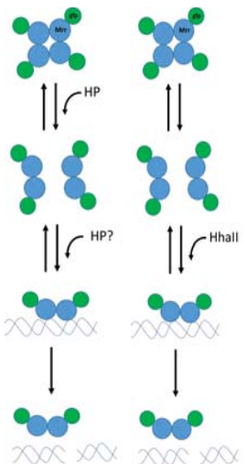
In vitro experiments: Gel filtration column (GF)



GF conclusion:

- **Confirmation of *in vivo* results that Mrr is a tetramer before HP shock.**
- **Mrr appears to be in equilibrium between dimers and tetramers.**
- **No large effect of pressure on the tetramer-dimer equilibrium after return to atmospheric pressure (data not shown) -reversibility is likely to be rapid *in vitro*.**

Resume:



⇒ Mrr inactive form is a tetramer.

⇒ HP shock could push the equilibrium towards the dimer, which is the active form.

⇒ Alternatively, pressure could modulate the chromosome structure, increasing the affinity of Mrr dimers and pulling the equilibrium toward the bound, active dimer. Since the cleavage is irreversible, then the Mrr dimer bound to DNA does not re-associate to tetramer after release of pressure.

⇒ HhaII is a methyl transferase. It is not present in *E.coli,* since this would be toxic for the cell. Indeed HhaII activity multiplies available sites for Mrr.

Expression of HhaII leads to methylation of DNA, an increase in affinity of the Mrr dimer for the now methylated DNA, hence pulling the equilibrium toward the bound and active dimer. Since the cleavage is irreversible, then the Mrr dimer bound to DNA does not re-associate to tetramer after release of pressure.

⇒ The Mrr dimer can bind the DNA and cut it with higher probability than the tetramer, probably due to a better accessibility of the binding domain and/or catalytic site.

Acknowledgments:

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