Measurement of intracellular metabolite pools upon methylglyoxal stress in *Escherichia coli* using LC-MS-MS.

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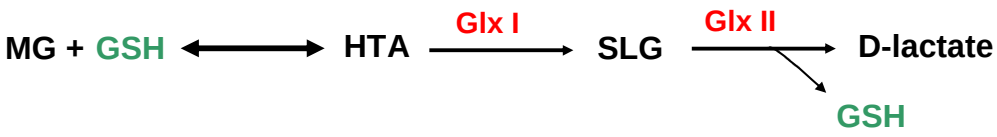
1. Introduction

Bacteria live in frequently changing environments and have to deal with a multitude of challenges. The chemical challenges to be faced are not only of exogenous origin, but can be the product of the metabolism, as in the case of methylglyoxal (MG), an endogenous electrophile that kills via damage to macromolecules. *Escherichia coli* can counteract the toxicity of MG. The glutathione-dependent glyoxalase system, consisting of glyoxalase I and II (Glx I & II), provides the main route for MG detoxification.

Protection from MG is highly dependent on the activity of the membrane proteins KefB and KefC. KefB and KefC are maintained inactive by the binding of glutathione (GSH), and are activated during MG detoxification by a specific intermediate molecule of the detoxification pathway, S-lactoylglutathione (SLG). The channels modulate the cytoplasmic ion composition (K^+, Na^+, H^+), which in turn leads to a cytoplasmic acidification. The degree of channel activation is affected by the relative concentrations of MG, GSH, and the specific activities of Glx I and Glx II leading to SLG. Therefore it is of interest to monitor changes in the concentration these metabolites during of detoxification in order to get a quantitative understanding of the process.



Described here is the procedure to measure the levels of GSH and SLG before and after exposure to MG using formic acid. A similar experimental set up as for potassium efflux experiments is used to which a silicon oil centrifugation step is incorporated. Subsequently, samples are analysed by LC-MS-MS in order to quantify intracellular GSH and SLG levels.

It is worth noting that GSH levels measured after exposure to MG are a composite of hemithioacetal (HTA) and GSH levels at the time of sampling. This is due to the chemical equilibrium and instability of HTA. The reverse reaction is strongly favoured as soon as MG is diluted due to cell lysis with formic acid.

Contents

2. MATERIALS AND EQUIPMENT REQUIRED

- 50ml Falcon tubes
- 20ml Universals tubes
- Microcentrifuge tubes
- 125ml conical flasks
- 37℃ shaking incubator
- 37℃ water bath
- Pipettes and Gilson pipettes
- Eppendorf Multipipette Plus
- Vacuum pump (Milipore, WP61 115 60)
- Flat forceps
- 0.45µm cellulose nitrate filters (Whatman, 47mm diameter; Cat.No. 7184-004)
- Timer
- Water jacketed stirring glass vessels with magnetic stirrer
- Aspirator
- Glass filter folder (Sartorius)

Chemicals and Biochemicals:

• $(NH_4)_2SO_4$	VWR – AnalR 100334C
• $K_2HPO_4 \cdot 3H_2O$	VWR – AnalR 103494G
• KH_2PO_4	VWR – AnalR 102034B
• $Na_2HPO_4 \cdot 12H_2O$	Fischer Scientific S/4360/60
• $NaH_2PO_4 \cdot 2H_2O$	Fischer Scientific S/3760/60
• KCl	VWR – AnalR 102034B
• Thiamine · HCl	Sigma, T-4625
• D-glucose	VWR – AnalR

- $MgSO_4 \cdot 7H_2O$ — Fischer Scientific M/1050/53
- $(NH_4)_2SO_4 \cdot FeSO_4 \cdot 6H_2O$ — VWR – AnalR 10022
- Tryptone — Oxoid LP0042
- Yeast extract — Oxoid LP0021
- Silicone oil AR20 — Fluka, 10836
- Silicone oil AP100 — Fluka, 10838
- Glu-Glu — Sigma, G3640
- Glutathione (GSH) — Sigma, G6529
- S-lactoylglutathione (SLG) — Sigma, L7140
- Formic acid (≥99% — Sigma, 251364
- Methylglyoxal (MG) — Sigma, M0252 (40% solution)
- N-ethylmaleimide (NEM) — Sigma, E3876

3. GROWTH MEDIA AND BUFFERS

Please refer to SOP for potassium efflux experiment for General growth procedures and media.

4. PROCEDURE FOR METABOLITE EXTRACTION

Preparations to be made 1 day in advance:

Prepare a silicone oil mixture a day before performing the experiment.

Silicone oil **A** (Fluka AR20)
Silicone oil **B** (Fluka AP100)

- mix oils A and B in a ratio of 3:2, i.e. for 6 mls of Fluka AR20 and 4 mls of Fluka AP100
- vortex briefly to mix oils and place in 65 C water bath for 1 h to allow proper mixing.
- let mixture oil cool down to room temperature on your bench

Growth of cells

1. Prepare overnight cultures by inoculating a single colony into 10ml of K0.2 medium in a 50 ml Falcon tube and incubate at 37℃ shaking at 200 rpm for at least 16 hours.

2. Next day measure the OD_{650} in a 1:10 dilution (in K0 buffer) to ensure that the cell culture has grown to the expected cell density.

3. Dilute the overnight culture 1/8 into fresh K0.2 medium.

    - grow a 30ml culture in a 125ml conical flask

    Note: Always grow a reference cell culture such as the wild type strain with a known behaviour in order to check for inconsistencies, to track possible errors or malfunctioning equipment.

4. Incubate at 37℃ and grow cells to an OD_{650} of ~0.8-1 (late logarithmic phase of growth).

    - Take OD readings to monitor growth but make sure not to take too many samples as 25ml of the culture are required for each experiment.

5. While cells are growing, prepare a solution of 2.5M formic acid containing 50µM Glu-Glu.

    - The Glu-Glu is an internal standard for the LC-MS-MS and is prepared from a stock solution (1mM in 1M formic acid) frozen at -20° C, i.e. dilute 1:20 into 2.5 M formic acid.

6. Aliquot 40µl of 2.5 M formic acid with Glu-Glu into microcentrifuge tubes

7. For each microcentrifuge tube, pipette 500µl of the silicone oil mixture on top of formic acid.

    - The formic acid and the silicone oil shouldn't mix and you should get two clear phases. Perform a quick 5 sec spin at 13000 rpm to check that the two phases really don't mix.

8. To collect samples, perform K^+ efflux experiment depending on the subject of investigation.

    - To extract GSH and SLG from cells, take 1ml cell suspension from efflux pots and apply on top of the silicone oil and perform a 30 sec spin at 14000rpm.

    - Check for separation of cells from medium into formic acid. Some cells may get stuck at the interphase between medium and silicone oil leading to errors.

    - The mixture of silicone oils used was chosen because it gave the most consistent separation of cell and medium.

9. Supernatant can be removed and formic acid recovered after completion of experiment.

    - aspirate the main part of the medium and the silicone oil, so that only a thin layer of silicone oil remains on top of the formic acid

    - perform a quick 5 sec spin to achieve a clean phase separation

    - take up formic acid with cell debris with a gel loading tip by going through the thin layer of silicone oil

    - spin sample at 4° C for 15 min at full speed to p ellet cell debris.

    - Take up supernatant and freeze a -20℃

Preparation of Standards for LC-MS-MS

- Take 40ul of S-LG (4.5mM in 1M FA) stock and 37.5ul GSH (4.8mM stock in water) and make up to 300ul with 2.5 M formic acid. This gives a stock containing 600uM of each compound.

- Prepare a 400µM Glu-Glu solution in 2.5 M formic acid

Prepare standard curves for GSH and SLG as follows and freeze together with samples at -20 C.

Standard	**Final conc (uM)**	**Vol 600uM stock (ul)**	**Vol 2.5 M formic acid (ul)**	**Vol 400µM Glu-Glu (ul)**
1	0	0	35	5
2	37.5	2.5	32.5	5
3	75	5	30	5
4	150	10	25	5
5	225	15	20	5
6	375	25	10	5
7	525	35	0	5

5. LC-MS-MS

The LC-MS-MS determination of GSH and SLG is performed at a central facility in Aberdeen (Gary A Cameron, Division of Applied Medicine, University of Aberdeen, UK) using a Thermo Surveyor-TSQ Quantum system with electrospray ionisation (ESI) in the positive ion mode. A Stability BSC 17 (5µ) column (150mmx2mm) was used and the analytes eluted with a mobile phase comprising, 50% 7.5mM ammonium formate, pH2.6 (formic acid) and 50% acetonitrile at a flow rate of 0.2ml/min. The column was maintained at 45℃. ESI conditions were as follows: spray voltage 4kV, sheath gas pressure 60, auxiliary gas 0 and capillary temperature 375℃. Detection was carried out in SRM mode at a collision pressure of 1.4 and a collision energy of 13V using the following SRM transitions: GSH m/z 308 – m/z 179, SLG m/z 380 – m/z 233 and Glu-Glu (internal standard) m/z 277 – 241. Quantification was carried out using Xcalibur software. All samples and standards were diluted 1:100 with water prior to injection (1µl) and were maintained at 4℃ in the autosampler.

6. DATA CONVERSION

The levels of GSH and SLG are determined using a standard curve and raw data are given in the unit µM. To obtain the approximate cytoplasmic concentrations of GSH and SLG, raw data are multiplied by a dilution factor due to the cell lysis in 40µl formic acid.
It is assumed that the cytoplasmic volume from a 1ml cell culture at OD_{650} 2 is equivalent to volume 1.6µl.

Data are transformed assuming OD 2=1.6µl of cytoplasm
Experiment performed with $8x10^8$ cells
$8x10^8$ cells = 0.64 µl of cytoplasm
0.64 µl in 40+0.64 µl is a dilution factor of 63.5
Data are calculated by multiplying raw data by the dilution factor 63.5