The Hong Kong University of Science and Technology

Department of Chemical and Biological Engineering

Undergraduate Research Opportunities Program:

Investigation of bacterial antibiotic persistence by proteomics

Ishita KUMAR (UG) - 20473100

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Supervisor: Prof. Henry Hei Ning LAM
Jordy Evan SULAIMAN

1. Abstract

Several relapsing infections are linked to bacterial persisters - a phenotype where a minority of the cells enter a dormant state and survive antibiotic treatments to then proliferate once the treatment has stopped. To study the resuscitation process, *E. coli* wild type and Δ*elaB* mutants cells were treated with ampicillin to isolate persisters. Samples of the persisters were allowed to resuscitate. Using shotgun proteomics, proteins expressed in the samples were identified. Although an insufficient amount of proteins were detected in the wild type cells, the investigation continued to compare the Δ*elaB* mutants cells. Fifteen differentially expressed proteins were found (7 up-regulated, 8 down-regulated). Studying the proteins showed that ribosomal proteins, chemotaxis, and aerobic respiration are important for persister resuscitation.

2. Introduction

Bacterial persisters cause recurring or chronic infections, such as tuberculosis, brucellosis, and typhoid fever, which affect millions of people every year [1][2]. Although first described just a few years after the discovery of antibiotics, research in this field has been sidelined to focus efforts on antibiotic-resistant bacteria [3][4]. Persisters showcase heterogenous behaviour in a bacterial population when subjected to stressful conditions, such as antibiotics. This subpopulation of the bacteria enters a dormant state which allows them to survive lethal doses of the antibiotic [5]. However, they are also capable of resuscitating and restoring the population when the antibiotic levels drop or the sources of stress are removed [5]. As more research is conducted on persisters, it is increasingly evident that this phenomenon is the result of several cellular stress responses that can advance adaptive evolution, which implies that persisters may behave as an intermediate for resistant bacteria [6]. Further insight into key mechanisms behind the formation and resuscitation of persisters could lead to more effective antimicrobial treatments against persister-related infections**.**

Persister behaviour is a phenotype expressed by the bacterial cells, they do not show any genetic differences in comparison to the susceptible cells [7]. Evidence of their growth arrest was seen when persisters were not killed by the antibiotic but also did not grow in its presence, unlike resistant bacteria. Therefore, it was hypothesised that persisters halt metabolic activity so that it cannot be targeted by the antibiotic, whereas resistant bacteria have a genetic mutation that blocks the antibiotic mechanism [7]. Dormancy in persisters has been linked to the toxin-antitoxin (TA) systems in the cell which are triggered by stress. The toxin is responsible for disturbing cellular processes which then lead to dormancy, and the antitoxin alleviates the toxicity. In *E. coli*, important TA systems include MazE-MazF, RelB–RelE, HipA–HipB, Phd–Doc, VapB–VapC and MqsR–MqsA [8]. Further research in *E. coli* persisters has highlighted the a decrease in ribosomal and carbohydrate metabolism proteins and an increase in membrane and transport proteins when persisters are formed [9][10].

It is important to note, however, that fewer investigations have been conducted on how persisters resuscitate, and the mechanisms that play a role in the process [11]. If treatments that could induce resuscitation of persister cells were developed, it would strengthen the effect of antibiotics. There are many aspects to consider, as described by *Jõers et al.*; when a persister is back in a stress-free environment they must first detect nutrients, resume their metabolic activity, change the cell's gene expression, and then continue cell growth [12]. Characterising the specific genes and proteins that play a role in these processes would provide a comprehensive idea of how persisters resuscitate and what we could target.

This study will utilise a proteomic analysis to delve deeper in this field of study. It has been seen that *E. coli* mutants lacking the *elaB* gene have increased persister cell formation, this is why this study will be conducted with the Δ*elaB* mutant to have a larger persister population to study [13]. Wild type cells will be used as a control. By doing a proteomics analysis of persister cells and their resuscitated progeny, differentially expressed proteins will be identified which will elucidate the persister resuscitation process.

3. Methods

Bacterial Strain and Growth Condition

The bacterial strain used for this investigation was *E. coli* K-12 MG1655, wild type (WT) and Δ*elaB* mutant. The samples were prepared by suspending the cells in Luria−Bertani (LB) broth at a 1:1000 ratio. They were incubated overnight at 37ºC at 220 rpm to obtain mid-exponential phase cultures.

Sample Preparation for Proteomics

Two samples types were prepared, one of the WT and Δ*elaB* mutant samples were to be persisters and the other two were to be the resuscitated progeny samples. The WT samples would act as a control. To isolate persisters, the mid-exponential phase cultures of *E. coli* (WT and Δ*elaB*) were treated with 3μL of ampicillin (100 μg/mL) and incubated for 1 hour at 37ºC and 220 rpm. The cultures were washed with LB broth immediately and samples were taken to be kept at -20ºC to terminate any further reactions. These samples would act as the WT and Δe*laB* samples that were not allowed to resuscitate. The remaining samples were once again incubated overnight at the same conditions to allow for the resuscitated progeny. The next day the resuscitated cells were washed with LB broth and also kept at -20ºC. Three biological replicates were made for each persister and resuscitated samples.

The samples were prepared for proteomics using filter-aided sample preparation (FASP) with Amicon ultra-0.5 centrifugal filter devices. The cell pellets of each sample were resuspended in lysis buffer (0.5% SDS, 50mM Tris-HCL (pH 8), 25mM dithiothreitol (DTT)) at a cell to buffer ratio of 1:10 and then incubated for five minutes at 95ºC. The samples were then briefly sonicated to reduce the viscosity of the lysate. Afterwards, they were centrifuged at 16,000 RCF for 5 minutes at 25ºC to remove cell debris and insoluble materials. To ensure the sample preparation was successful and to gain more insight into the amount of proteins present, a BCA protein assay was done with each sample using a Pierce BCA protein assay kit. Next, to remove SDS, 50μL of the cell lysate was mixed with 250μL of exchange buffer (6 M

urea, 50 mM Tris-HCl (pH 8), 600 mM guadinine HCl), and then put through a Amicon filter device and centrifuged at 14,000 RCF for 20 minutes at 25ºC. Then 250µL of exchange buffer was added again, centrifuged at the same conditions, after which the filtrate was discarded. The proteins, now in the filter, were subject to alkylation with 2-iodoacetamide (IAA) solution (50 mM IAA in exchange buffer) in the dark for 20 minutes and centrifuged. The filtrate in the collection tube was discarded, 250µL of 50 mM ammonium bicarbonate was added and the samples were centrifuged (this step was repeated). Finally, for tryptic digestion, sequencing-grade-modified trypsin (1:100 w/w) was added with 250µL of 50 mM ammonium bicarbonate and the samples were incubated for 12 hours at 37ºC. The filtrate of the samples were acidified with 10% formic acid and centrifuged again. The samples were desalted using C18 ZipTips. Lastly, the samples were made to dry in a SpeedVac and kept at −20°C.

Liquid Chromatography−Mass Spectrometry (LC-MS)

The samples were processed using shotgun proteomics techniques, an ultra-performance liquid chromatography to quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS) was used. A '.raw' file was received for each sample from the UPLC-Q-TOF-MS.

Sequence Database

The *E. coli* K-12 protein sequence database (a '.fasta' file) was acquired from the UniProt database. The '.raw' files of each sample from the UPLC-Q-TOF-MS was first converted to '.mzXML' files using the ProteoWizard software (MSConvert) to visualise the peaks of each sample as detected by the LC-MS [14]. Then, a target decoy database was added using the *E. coli* K-12 protein sequence database by shuffling peptide sequences but keeping the tryptic amino acids K and R fixed [15]. The ratio of real spectra to the decoy sequences was 1:10. The decoy sequences are made to approximate the false discovery rate (FDR) of the identified protein sequences. The '.mzXML' files were put through Comet and then Xinteract (a Trans-Proteomics Pipeline (TPP) which includes PeptideProphet, iProphet, and ProteinProphet) [16] [17]. The files

obtained from Xinteract were then turned into Excel files for easier analysis and visualisation. The threshold to keep the FDR at <1% was found for each sample using Xinteract, and the detected proteins were filtered accordingly.

Label-Free Quantification by Spectral Counting and Bioinformatic Tools

To obtain a comprehensive protein identification list of for each sample type, proteins that were detected in two or three of the biological replicates were counted. Once the common list of proteins was found for each sample type, these proteins were quantified by using the 'total independent spectra' count to calculate each protein's corresponding normalised spectral abundance factor (NSAF) in each replicate [18]. It is calculated using the formula below:

$$(\mathrm{NSAF})_J = \frac{(\mathrm{Sc}/L)_J}{\sum_{i=1}^{N} (\mathrm{Sc}/L)_i}$$

where Sc is the 'total independent spectra' count of protein *J*, *L* is the length of protein *J,* and *N* is the total number of proteins identified [18]. The average of each replicate's NSAF was the protein's overall NSAF for the sample type. Next, the proteins that were common between the persister sample and the corresponding resuscitated progeny sample were identified, these proteins were considered for differential protein expression. To find significantly differentially expressed protein, the list of proteins was further refined based on the protein's p-value from the Student's t-test (paired, two-tailed) on the NSAF values being lower than 0.05, and if the fold change was higher or lower than ±1.5. A volcano plot was made to find the significantly differentially expressed proteins. Finally, to find interactions and connections between the chosen differentially expressed proteins, STRING (version 11.0) was used to showcase the network [19].

4. Results

After obtaining the results, it was seen that a sufficient amount of proteins had not been detected in the WT samples (persister and resuscitated progeny). It is believed to be due to two reasons - there were not

enough cells after antibiotic treatment (with regards to the BCA assay, DTT interference indicated there was sufficient amount of proteins), and the lysis buffer used in the FASP method for proteomics preparation was not effective. The results discussed in this paper will be focused on the comparison between the Δ*elaB* mutant persisters and its resuscitated progeny.

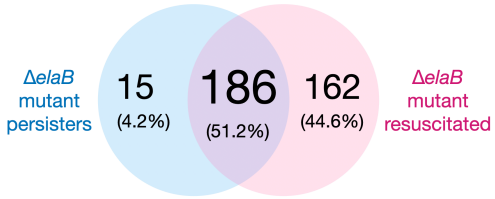

Figure 1 - Number of common and uncommon proteins identified between ΔelaB *mutant persisters and their resuscitated cells*

The Δ*elaB* mutant persisters and their resuscitated cells showcased 201 and 348 proteins respectively. When the identified proteins were compared to one another (*Figure 1*), 186 proteins were seen to be common between both sample types. Fifteen proteins (4.2%) were found to be unique in the Δ*elaB* mutant persisters, whereas in the resuscitated cells there were 162 (44.6%). These results follow the trend seen by similar studies, protein synthesis in persisters is found to be considerably low when compared to actively growing bacteria, as seen by the 162 new proteins that the resuscitated cells express [20]. As mentioned earlier, the common proteins were further analysed for differential expression. The results can be seen in *Figure 2*. The proteins with a p-value lower than 0.05, and fold change above or below than ±1.5 were considered to be differently expressed. Out of all the 186 common proteins, 15 proteins were seen to be expressed differentially, with 7 proteins being up-regulated and 8 being down-regulated.

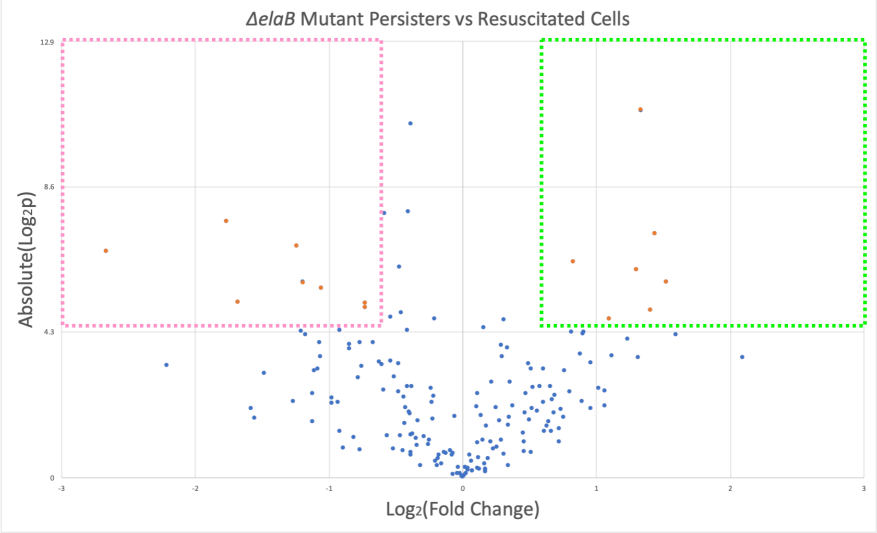

Figure 2 - Volcano plot for ΔelaB *mutant persisters and their resuscitated cells. The highlighted proteins are differentially expressed (defined as p-values lower that 0.05 and fold change higher or lower than ±1.5), the proteins in the green box are up-regulated and proteins in the pink box are down-regulated.*

The 15 proteins that were differential expressed can be seen in *Table 1*. Ribosomal and amino acid biosynthesis proteins were up-regulated in resuscitated cells. This is consistent with the current understanding since these proteins are down-regulated in persisters to induce a dormant state [9]. Once the cell starts to resuscitate, ribosomal and amino acid biosynthesis protein expression must be increased for increased protein synthesis. In addition to this, several proteins involved in oxidative damage repair were also up-regulated. Most of the down-regulated proteins were ones involved in oxidative stress defence, sugar transport, and anaerobic respiration. These are all essential processes that *E. coli* persisters implement in response to stresses such antibiotics [21].

Table 1 - List of differently expressed proteins and their corresponding genes, between ΔelaB *mutant persisters and their resuscitated cells, along with protein regulation*

Gene	Protein	Regulation	p-value	Fold Change
iscS	cysteine desulfurase	Up-regulated	0.03919	2.14
deoD	purine nucleoside phosphorylase (PNP-I)	Up-regulated	0.01836	2.87
malE	maltose/maltodextrin-binding periplasmic protein	Up-regulated	0.01228	1.77
typA	50S ribosomal subunit assembly factor BipA	Up-regulated	0.03271	2.65
rplE	50S ribosomal protein L5	Up-regulated	0.00054	2.52
rplJ	50S ribosomal protein L10	Up-regulated	0.01434	2.46
fusA	elongation factor G (ribsomal translocation)	Up-regulated	0.00685	2.70
frdA	fumarate reductase flavoprotein subunit	Down-regulated	0.01855	0.44

guaB	inosine-5'-monophosphate dehydrogenase	Down-regulated	0.03107	0.60
gatY	D-tagatose-1,6-bisphosphate aldolase subunit GatY	Down-regulated	0.00877	0.42
grcA	autonomous glycyl radical cofactor (YfiD)	Down-regulated	0.00987	0.16
crr	PTS system glucose-specific EIIA component	Down-regulated	0.02854	0.60
ahpF	alkyl hydroperoxide reductase subunit F	Down-regulated	0.02095	0.48
ihfA	integration host factor subunit alpha	Down-regulated	0.02788	0.31
potA	spermidine/putrescine import ATP-binding protein PotA	Down-regulated	0.00537	0.29

5. Discussion

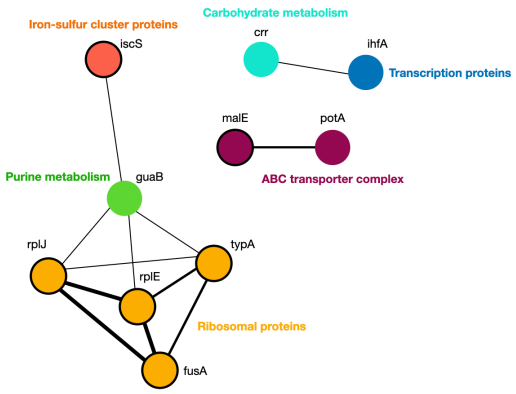

Figure 3 - Differentially expressed proteins for ΔelaB *mutant persisters and their resuscitated cells which have protein-protein interactions as indicated by STRING (version 11.0). The thickness of lines indicate the confidence of interaction. Proteins outlined in black are up-regulated, and not outlined are down-regulated.*

Higher Ribosomal Proteins Levels in Resuscitated Cells

In *Figure 3*, the protein-protein interactions found among the differently expressed proteins was visualised using STRING (version 11.0). One of the most up-regulated pathways is ribosomal protein interactions, where all four proteins were up-regulated by around 2.5-fold. This observation reinforces the trends seen in the literature; persister cells are known to down-regulate ribosomal and protein synthesis activities in order to reach a dormant state, and to be able to resuscitate again ribosomal protein expression would have to be increased [9][22]. *Yamasaki et al.* reached a similar conclusion after they saw that higher levels of the ribosome resuscitation factor, HflX, is one of the key factors behind faster persister cell waking [23]. In conjunction with this, the purine nucleoside phosphorylase protein (PNP-I) was also up-regulated. PNP-I functions to produce bases for the synthesis of nucleotides [24]. Cellular ribosomal protein expression

regulates nucleotide biosynthesis, providing further evidence that suppressing protein synthesis and ribosomal proteins leads to persister formation but increasing levels encourages resuscitation [25].

Protein for Oxidative Repair Damage Up-Regulated

Up-regulation of cysteine desulfurase (IscS) was found to be of interest. This protein plays a major role in the repair of iron-sulfur clusters that are damaged by oxidation [26]. Ampicillin has been known to cause oxidative damage in bacteria by increasing levels of hydrogen peroxide, and the up-regulation of IscS is another strong indicator [21]. Iron-sulfur cluster assembly is important for the biosynthesis of a large range of proteins, which highlights the previous discussion point about the need of protein synthesis for persister resuscitation [26]. This may be a reason why iron-sulfur cluster binding proteins have been seen to be down-regulated in persisters [10].

Chemotaxis and Low cAMP Levels Required for Resuscitation

MalE, part of the ABC transporter complex for maltose and maltodextrin import, was up-regulated in the resuscitated progeny. This protein, when bounded to substrate, can also bind with the chemoreceptor, Tar, which prompts chemotaxis towards maltose [27]. This is once again mirrored in the study by *Yamasaki et al.* where it was concluded that chemotaxis proteins like Tar are needed for persister resuscitation [23]. Another interesting observation was the down-regulation of the EIIA protein. This protein when down-regulated, has been observed to reduce cyclic adenosine monophosphate (cAMP), which then accelerates persister cell waking [23].

Oxidative Stress Response Proteins Down-Regulated

Four proteins involved in oxidative stress response in *E. coli* were down-regulated in the resuscitated cells, they include GatY, YfiD, AhpF, and PotA. GatY plays a role in the import of alternative carbon sources, like galactitol, and known to be expressed when the cell is affected by oxide stress [28][29][30].

YfiD is required for an alternate form of pyruvate formate-lyase (PFL) when it is damaged under stress conditions [31]. Interestingly, it was found that MazF's (toxin of an *E. coli* TA system) activation is the cause of heterogenous expression of *grcA*, which encodes for Yfid [32]. This down-regulation of Yfid in the resuscitated cells implies that the MazE-MazF toxin-antitoxin system is involved in the persister formation. Next, AhpF is also known to be induced by oxide stresses and protects cells from damage by alkyl hydroperoxides [33][34]. The implications of the down-regulation of these proteins is also validated by the observations of *Sulaiman et al.*, where YfiD and AhpF were significantly up-regulated in *E. coli* persister cells [35]. Lastly, PotA is part of the ABC transporter complex in spermidine/putrescine import [36]. These polyamines protect *E. coli* cells from oxide damage and when in excess allow for retardation of protein synthesis, possibly to reach a dormant state [37]. It is worthy to note that such a wide range of oxidative stress responses were employed in the persister cells in response to the ampicillin, and were then down-regulated when the stress was removed. Considering ElaB provides resistance to oxidative stress, further studies should be done with a WT control to assess if these proteins compensate for the lack of *elaB* [13].

Persister Cells Undergo Anaerobic Respiration to Survive Ampicillin Treatment

The down-regulation of both the fumarate reductase flavoprotein subunit and YfiD indicate that persister cells may be resorting to anaerobic respiration to survive against ampicillin. The fumarate reductase flavoprotein is an enzyme required for anaerobic respiration, and YfiD is normally detected at high levels in anaerobic bacteria which have PFL [38][39]. Antibiotics like ampicillin lose their effectiveness when used in anaerobic conditions, the possible adaptation by persister cells to anaerobically respirate provides further evidence of this [21].

Integration Host Factor Down-Regulated

Not much is known about the function of the Integration Host Factor (IHF) protein; it has been vaguely associated with altering gene expression by transcriptional control [40]. The lack of understanding

makes it difficult to suggest why it has been down-regulated in the resuscitating cells. This study believes it plays in a role in changing the proteins expressed in persister and resuscitating cells.

6. Conclusion

To study the process of persister resuscitation, *E. coli* Δ*elaB* mutant cells were treated with ampicillin to augment persister cells. Samples of the cells were then put in conditions to facilitate resuscitation. The FASP method was used to prepare the samples for shotgun proteomics with a UPLC-Q-TOF-MS. A total of 201 and 348 proteins were found in the Δ*elaB* mutant persisters and resuscitated cells respectively, out of which 186 were common between them. Using various bioinformatic tools, differentially expressed proteins between the Δ*elaB* mutant persisters and resuscitated cells were identified. Fifteen proteins were found, where 7 were up-regulated and 8 were down-regulated. A thorough study of each of the proteins concluded persister resuscitation is dependent on aerobic respiration, an increase in ribosomal proteins, along with transporters and chemotaxis. An interesting avenue of study would also be to study the oxidative stress responses that the cell utilises or overproduces in the absence of ElaB. However, for this further research it is important to have a WT control with sufficient protein expression, which is why an alternate lysis buffer will be used in the FASP method in the future. In conclusion, this research project gave a much needed insight into the persister resuscitation process.

7. References

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