Chapter 5

"DELAYED GROWTH STASIS "
IN S.cerevisiae UPON
GLUTATHIONE DEPLETION:
DESCRIPTION AND
INVESTIGATION OF THE
PHENOMENON
5.1: Introduction

As described in general introduction (Section 1.1), glutathione (γ-glutamyl-cysteinyl glycine), the most abundant, non-protein thiol compound present in almost all eukaryotic cells and many prokaryotes, plays numerous roles in the cell, including protection from oxidative stress, drug detoxification, maintenance of the redox environment, assisting in protein folding, and storage of sulphur.

Although glutathione plays numerous roles within the cell, studies in *E. coli* have shown that is not essential for growth in *E. coli*, since mutants disrupted in glutathione biosynthesis are viable and grow well (Murata and Kimura, 1982). Earlier studies with *Saccharomyces cerevisiae* glutathione biosynthetic mutants had indicated that these mutants could grow in the absence of glutathione (Ohtake *et al.*, 1990), however subsequent studies have demonstrated that glutathione biosynthesis is in fact essential for the yeast *S. cerevisiae* (Wu and Moye-Rowley, 1994, Grant *et al.*, 1996) and *Schizosaccharomyces pombe* (Chaudhuri *et al.*, 1997), since the disruption of the first enzyme in glutathione biosynthesis results in glutathione auxotrophy in both organisms as seen on plates.

During the course of our investigation on the role of glutathione in *S. cerevisiae* and *S. pombe*, we observed that disruption of glutathione biosynthesis leads to glutathione auxotrophy in both organisms as seen on plates. Yet, in liquid medium there was apparently no effect of glutathione depletion on the growth of *S. cerevisiae* until the end of the growth curve. In contrast, glutathione depletion in liquid culture rapidly induced growth stasis in *S. pombe*. This phenomenon was investigated in detail and the investigation and description of this phenomenon is described in this chapter.

5.2: Growth behavior of *gsh1Δgcs1Δ* strains of *S. cerevisiae* and *S. pombe* on solid medium vs. liquid medium.

During the course of our investigation on the role of glutathione in *S. cerevisiae* and *S. pombe*, we observed that disruption of glutathione biosynthetic enzyme leads to glutathione auxotrophy in both organisms as seen on plates (Fig. 5.1). This has been described earlier by other workers also (Wu *et al.*, 1994, Grant *et al.*, 1996, Chaudhuri *et al.*, 1997). However
Fig. 5.1: (A) *S. cerevisiae* gsh1Δ cells and (B) *S. pombe* gcs1Δ cells showing glutathione auxotrophy on plates lacking glutathione.
when these cells were grown in liquid medium lacking glutathione, a rapid growth inhibition was observed with *S. pombe*, but in sharp contrast, *S. cerevisiae gsh1Δ* cells continue to grow until the end of growth curve (Fig. 5.2). This suggested that there was apparently no effect of glutathione depletion on the growth of *S. cerevisiae gsh1Δ* cells. Thus, although the behaviour of *S. pombe gcs1Δ* mutants on plates was reflected in liquid medium also, the behaviour of *S. cerevisiae gsh1Δ* mutant was unusual, showing auxotrophy on plates, but no apparent auxotrophy in broth.

5.3: The unusual growth behavior of *S. cerevisiae gsh1Δ* cells is a result of "delayed growth stasis".

One possible explanation for the different growth patterns observed in liquid medium as compared to plates is that *S. cerevisiae gsh1Δ* cells undergoes delayed growth stasis which is not apparent during the 6-7 generations corresponding to the full period of the growth curve in glutathione-free medium. To check if this is the case, *S. cerevisiae gsh1Δ* cells were allowed to grow in glutathione free medium till the end of the growth curve and were then re-inoculated in fresh glutathione free medium. After this subsequent reinoculation, the cells gradually entered into the growth stasis (Fig. 5.3). To test whether this was exclusively occurring on liquid medium or was also occurring on solid medium, the plates were examined for the presence of micro-colonies. These micro-colonies would represent small bunch of cells corresponding to the growth for a limited numbers of generations. Such small colonies would not be visible to the naked eye and would appear as glutathione auxotrophs on plates. Plates containing *gsh1Δ gcs1Δ* cells were streaked out on minimal medium lacking glutathione. Micro-colonies were observed with *S. cerevisiae* but not with *S. pombe* confirming our results with liquid medium (Fig. 5.4).

5.4: Growth of *S. cerevisiae gsh1Δ* and *S. pombe gcs1Δ* on GSSG vs. GSH.

One of the reasons for the "delayed growth stasis" observed in *S. cerevisiae* upon glutathione depletion could be accumulation of larger amounts of glutathione in the preinoculum phase than *S. pombe*. Since *S. cerevisiae* has been shown previously to utilize oxidized glutathione (Schmidt *et al.*, 1996), it is possible that storage pool of GSH is
Fig. 5.2: Growth of *S. cerevisiae gsh1Δ* and *S. pombe gcs1Δ* in minimal medium with (+) or without (-) glutathione (+/- GSH).
Fig. 5.3: *S. cerevisiae gsh1Δ* strains grown in glutathione-free medium for 16 hours followed by re-inoculation into +glutathione, -glutathione medium.

![Figure 5.3: Growth curves for *S. cerevisiae gsh1Δ* strains.](image)

**S. pombe**  **S. cerevisiae**

Fig 5.4: Formation of microcolonies by *S. cerevisiae gsh1Δ* cells (as seen under microscope) in glutathione free plates.

![Figure 5.4: Microcolonies by *S. cerevisiae gsh1Δ* cells.](image)
converted to oxidized glutathione during growth under aerobic conditions especially in liquid cultures, which are strongly aerated. This oxidized glutathione may be more efficiently absorbed in *S. cerevisiae* than in *S. pombe*. This possibility is also suggested by the fact that plant cells preferentially transport oxidized glutathione (GSSG) than reduced glutathione (GSH) (Jamai *et al.*, 1996). This would increase the storage pool available to resist glutathione depletion in *S. cerevisiae*.

To check this possibility, *S. cerevisiae gsh1Δ* and *S. pombe gcs1Δ* cells were grown in reduced glutathione and oxidized glutathione. However we observed that under these conditions, the growth patterns on both the oxidized and reduced glutathione were not dramatically different in either of the yeasts (Fig. 5.5). These results indicated that both species could utilize oxidized as well as reduced glutathione efficiently as a source of glutathione.

5.5: Comparison of storage and depletion of glutathione in *S. cerevisiae* and *S. pombe*.

Since we couldn’t find any difference in utilization of both the oxidized and reduced forms of glutathione by both *S. cerevisiae* and *S. pombe*, we decided to examine if there were any differences in the storage of glutathione, as well as if there was any difference in the rates of turnover of glutathione. Differences in either the storage or the rates of glutathione turnover could also account for the differential responses to glutathione depletion in the two yeasts. The experiments were carried out in cells that were deficient in GSH biosynthesis (*gsh1Δgcs1Δ*). Cells were grown in glutathione containing minimal medium, and after washing were transferred to glutathione free medium. At different time intervals the total glutathione levels were measured. The results of the glutathione depletion in the absence of ongoing glutathione biosynthesis are shown in Fig. 5.6. Interestingly, and in contrast to what we expected, we could observe neither a significant difference in the storage levels of glutathione in *S. cerevisiae* as compared to *S. pombe* nor in the rate of depletion of the glutathione that could have accounted for the delayed growth stasis phenotype that we were observing.
Fig. 5.5: Comparison of growth on oxidized (GSSG) vs. reduced (GSH) glutathione. Strains were grown in glutathione-free medium for 20hrs followed by re-inoculation into the indicated medium.

Fig. 5.6: Intracellular glutathione levels in (A) *S. cerevisiae* and (B) *S. pombe* grown in minimal medium and then reinoculated in glutathione free medium after washing.
5.6: Thioredoxin can partially complement GSH auxotrophy in *S. cerevisiae* but not in *S. pombe*.

A previous study has indicated that *S. cerevisiae* glutathione auxotrophs (gsh1Δ) can grow on medium containing other sulphydryl compounds like DTT, β-mercaptoethanol or cysteine. It was therefore possible that some sulphydryl compound, such as cysteine (Grant *et al.*, 1996), present in the cell might be compensating partially for glutathione auxotrophy. However previous work in our lab on supplementation of gsh1Δ mutant with cysteine did not permit growth of these mutants in contrast to earlier studies (Grant *et al.*, 1996). The reasons for the differences observed with previous workers are not clear. We subsequently decided to examine if yeast thioredoxin might be an endogenous sulphydryl compound that was partially compensating for glutathione depletion in yeast and be responsible for the phenomenon of delayed growth stasis upon glutathione depletion. We cloned yeast thioredoxin gene, TRX1, by PCR and expressed it downstream of a strong promoter, TEF1 (Mumberg *et al.*, 1995), on a single copy vector, and also from stronger GPD promoter on a multicopy plasmid (Mumberg *et al.*, 1995). These TRX1 containing plasmids when transformed in yeast gsh1Δ cells were able to compensate for glutathione depletion to a level where we could see small colonies appearing on plates lacking glutathione (Fig. 5.7). Although the size of colonies were bigger in case of expression from GPD promoter, but the complementation was still partial since after a certain size the colonies failed to grow. In contrast the transformants containing GSH1 gene on an overexpressing plasmid continued to grow (Fig. 5.8). These results suggested an increased delay in the onset of growth stasis by overexpression of thioredoxin, which was probably a consequence of the ability of the elevated levels of thioredoxin to cause a relief from glutathione depletion. The complementation by TRX1, was however, partial.

Since *S. pombe* failed to display any delayed growth stasis upon glutathione depletion, we decided to examine if thioredoxin overproduction can induce some delayed growth stasis in *S. pombe*. The yeast TRX1 gene was subcloned into a yeast expression vector pART1. *S. pombe* gcs1Δ cells were transformed with this plasmid containing thioredoxin downstream of the *S. pombe* ADH1 promoter and the transformants were directly selected on glutathione-deficient medium. However, no detectable colonies were observed, suggesting that
Fig. 5.7: Partial complementation of glutathione auxotrophy of gsh1Δ of *S. cerevisiae* by plasmid pTEF-TRX1 gene grown in SD-GSH plates.

Fig. 5.8: *S. cerevisiae* gsh1Δ cells transformed with pTEF-TRX1, pGPD-TRX1, pTEF-GSH1 yield microcolonies that do not grow further upon being restreaked on medium w/o glutathione.
thioredoxin over-production could not induce any significant levels of 'delayed stasis' in S. pombe. To confirm the expression, we also selected for transformants on MM-Leu + GSH medium and expression of the thioredoxin gene was confirmed by RT-PCR.

5.7: 'Delayed growth stasis' of S. cerevisiae gsh1Δ cells is not due to its petite-positive nature.

An earlier study has demonstrated that S. cerevisiae cells grown under GSH deficiency give rise to respiration deficient rho- cells (Schmidt, 1996). These mutants are deficient in mitochondria and are referred to as petites. We re-examined the effect of glutathione deficiency on the frequency of petite formation of cells and compared it to WT cells (Table 5.1). These results, shown in Table 5.1, clearly indicate an increased petite formation in gsh1Δ cells highlighting the importance of GSH in mitochondrial function and maintenance.

To check if the ability to form petites (petite positive nature) is responsible for the phenomenon of 'delayed growth stasis', S. cerevisiae gsh1Δ cells were grown in glycerol medium, a condition which requires functional mitochondria, so that there is no mitochondrial loss. The cells were reinoculated after washing into the same medium with or without GSH. As shown in Fig. 5.9, S. cerevisiae gsh1Δ cells in the absence of GSH show a much reduced 'delayed growth stasis'. Although growth is slow, cells do not stop growing even after 40 hr. Thus, although there is still a delay in the onset of growth stasis, it is far less as compared to growth on glucose medium. We believe this to be due to increased requirement of GSH in the mitochondria.

5.8: Thioredoxin over-expression can delay growth stasis in glycerol-grown glutathione-depleted cultures of S. cerevisiae.

We have found that the delayed growth stasis observed upon GSH depletion in S. cerevisiae was far less apparent in glycerol-grown cells. It was therefore necessary to determine if thioredoxin overproduction can lead to an increased delay in growth stasis even in glycerol-grown cells. S. cerevisiae gsh1Δ cells were transformed with the thioredoxin
Table 5.1: Frequency of petite formation in WT vs. gsh1Δ cells of S. cerevisiae.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Total no. of cells</th>
<th>No. of petites</th>
<th>% of petites</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>1816</td>
<td>221</td>
<td>12.16%</td>
</tr>
<tr>
<td>WT</td>
<td>2463</td>
<td>345</td>
<td>14.0%</td>
</tr>
<tr>
<td>gsh1Δ</td>
<td>1781</td>
<td>493</td>
<td>27.68%</td>
</tr>
<tr>
<td>gsh1Δ</td>
<td>2574</td>
<td>783</td>
<td>30.5%</td>
</tr>
</tbody>
</table>
over-producing plasmid, and transformants were selected directly on plates lacking GSH. After 5-7 days of growth, we could observe small colonies appearing on these plates as opposed to the same cells transformed with control vectors (Fig. 5.10). This indicates that although there is a far reduced delayed stasis phenomenon in S. cerevisiae glycerol-grown cells, the stasis can still be further delayed by overproducing thioredoxin, a phenomenon that we previously observed in the glucose-grown cells.

5.9: S. pombe petite positive mutant (ptpl-1) in gcs1Δ background also showed rapid growth stasis upon glutathione depletion like gcs1Δ cells.

Unlike S. cerevisiae, S. pombe is petite negative yeast. These yeasts are unable to show up the cells with dysfunctional mitochondria. It might be possible, therefore that S. pombe if made to lose its mitochondria could undergo 'delayed growth stasis', since it could behave like S. cerevisiae under these conditions. We thus examined if petite positive mutants of S. pombe might be able to display delayed growth stasis. S. pombe petite positive mutants (ptpl-1) were obtained from Dr. J. L. Paluh. The ptpl-1 mutant contains a mutation in PTP1 gene, which leads to total loss of mitochondrial DNA. This leads to a petite positive phenotype in S. pombe, although no other phenotypes were associated with ptpl mutation in rho+ strains (Haffter and Fox, 1992). A gcs1Δ was introduced in this strain to yield gcs1Δ ptpl-1 strain. This strain was grown in minimal medium containing glutathione and reinoculated into glutathione free medium to examine if it displays a 'delayed growth stasis' phenotype. However, the observation in these strains were similar to S. pombe gcs1Δ strains described earlier, and the petite positive mutation did not appear to confer any significant advantage to S. pombe gcs1Δ to grow under GSH depletion conditions.

5.10: Discussion

In this study we have described the phenomenon of 'delayed growth stasis' that results from glutathione depletion in S. cerevisiae and tried to elucidate its mechanism by studying various aspects of glutathione metabolism. We have tried to resolve the conflicting reports regarding the nature of glutathione auxotrophy shown by S. cerevisiae gsh1Δ in liquid culture.
Fig. 5.9: Growth of *S. cerevisiae gsh1Δ* strains on glycerol.

Fig. 5.10: *S. cerevisiae gsh1Δ* cells transformed with (A) pTEF-416 (B), pTEF-TRX1. Transformants were selected on SD (glycerol)-URA-GSH. Growth was recorded after 5 days.
(Ohtake, 1990; this study) and in plates (Wu & Moye-Rowley, 1994; Grant et al., 1996, Chaudhury et al., 1997).

We have shown that upon glutathione depletion S. cerevisiae gsh1A cells can grow for about 7-8 generations without its growth rate being affected, leading to apparent growth of S. cerevisiae gsh1A in cultures. The formation of microcolonies on plates further indicated that this phenomenon was also seen in plates, although not discernable to naked eyes.

We have examined various aspects of glutathione metabolism in S. cerevisiae and have also compared it with S. pombe, which does not display the phenomenon of 'delayed growth stasis'. To explain why S. pombe is behaving differently than S. cerevisiae upon glutathione depletion, we compared the various aspects of glutathione metabolism in both yeasts to see if they might explain the differential behaviour of these two yeasts upon glutathione starvation. However,

we did not find any significant difference in the metabolism of glutathione in both yeasts.

We also examined if the petite-positive nature of S. cerevisiae versus the petite negative of S. pombe might be an explanation for this phenomenon. The petites have defect in mitochondria. A petite positive yeast, such as S. cerevisiae, is one that can give rise to petite colonies lacking functional mitochondria, while a petite negative yeast cannot give rise to such colonies since a functional mitochondria is essential in these yeasts. These defects or mutations are not lethal because the cell can grow (more slowly) by fermentation, deriving energy from just the initial steps of sugar metabolism, converting six-carbon sugars to three carbon products, such as glycerol. To use glycerol, functional mitochondria are required which is why petites are unable to grow on glycerol as the sole source of carbon.

Mitochondria are the sites of vital cellular functions such as aerobic respiration (oxidative phosphorylation). During respiration, incomplete reduction of dioxygen results in the formation of reactive oxygen intermediates. These intermediates are highly reactive and can lead to lipid peroxidation, protein inactivation and DNA breakage.

A major function of glutathione is to protect cells from these reactive oxygen species, which can cause oxidative stress in cell. In conditions where glutathione is limiting, loss of
mitochondria can help cell to cope up better with the situation, thus providing more chance of survival.

Since *S. cerevisiae* exhibit 'delayed growth stasis' even when grown in glycerol medium it indicates that the petite positive nature of *S. cerevisiae* is not a true explanation for 'delayed growth stasis'. Furthermore, the inability of *S. pombe* gcs1Δ cells to show this phenomenon even in a petite-positive background indicates that this is not an explanation for the difference in behaviour.

Overexpression of thioredoxin however, could significantly increase the delay in onset of growth stasis in *S. cerevisiae* but not in *S. pombe*. In this context it is interesting to note that *S. cerevisiae* glutathione reductase disruptants are viable and are non-viable only when both TRX1 and TRX2 are also disrupted (Muller, 1995, 1996), while disruption of glutathione reductase is non-lethal in *S. cerevisiae*, it is lethal in *S. pombe* (Lee *et al.*, 1997). In both prokaryotes and eukaryotes two pathways exist within the cell for the reduction of the protein disulfide bonds in the cytosol. One is glutathione-glutathione reductase-glutaredoxin system and other is thioredoxin-thioredoxin reductase pathway. Although both systems use NADPH for their reducing power, they are separate pathway since neither glutathione efficiently reduces the thioredoxins, nor can thioredoxin reductase efficiently reduce the glutaredoxins (Prinz, 1997). Genetic and biochemical studies with *E. coli* have, however, recently shown that there is a somewhat complex intersection of these pathways. While there is specificity in the two pathways, there is also a partial redundancy as well (Miranda-Vizuete, 1996; Prinz, 1997; Aslund & Beckwith, 1999).

Although both *S. cerevisiae* and *S. pombe* appear to be similar as far as metabolism of glutathione is concerned, but still TRX1 is not able to cause delayed growth stasis in *S. pombe*. However, the rapid growth stasis that ensues upon glutathione depletion does not necessarily imply that there is no intersection of these two pathways in *S. pombe*. Rather, it only indicates that some essential enzyme in *S. pombe* has an absolute requirement for glutathione that cannot be replaced by thioredoxin.