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COMMUNICATION

Iterative synthetic strategies and gene deletant experiments enable the first identification of polysulfides in *Saccharomyces cerevisiae*

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Lisa I. Pilkington,†[a] Rebecca C. Deed,[a,b] Katie Parish-Virtue,[a] Chien-Wei Huang,[a] Michelle E. Walker,[c] Vladimir Jiranek,[c] David Barker,†[a] Bruno Fedrizzi†[a]

New evidence on the role of H_2S as a gasotransmitter suggests that the true signalling effectors are polysulfides. Both oxidized polysulfides and hydropolysulfides were synthesized and their presence in *S. cerevisiae* was observed for the first time. A single gene-deletant approach allowed observation of the modulation of polysulfide species and levels.

Hydrogen sulfide (H_2S) has been postulated to act as an important molecule in signaling and hormone regulation in prokaryotes and eukaryotes.[1-5] H_2S influences physiological functions in various biological systems, including cardiovascular, neurotransmission, vasodilation, apoptosis and immunity.[6] Despite evidence that H_2S acts as signaling species, the mechanisms by which this molecule acts are still unknown and/or contentious.

Recent studies have shown the critical role of polysulfides in oxidative stress and cell signaling (Fig. 1);[7-10] polysulfides appear to produce H_2S as a degradation product and could act as the signaling species, rather than H_2S. These claims, however, are putative and there are very few studies providing evidence for polysulfides in this role.

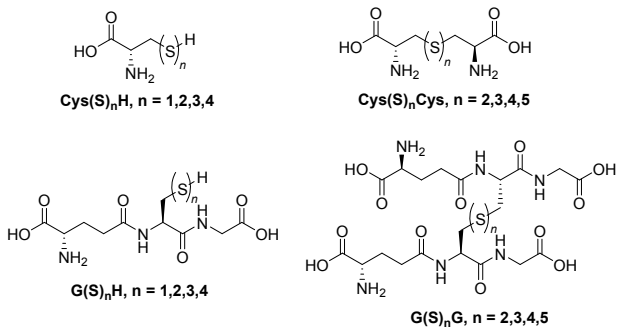

Figure 1: Polysulfides of interest; oxidized polysulfides: $R(S)_nSR$ and hydropolysulfides: $R(S)_nH$, where *R* is an organic moiety, i.e. glutathione (G) and cysteine (Cys), and $2 \leq n \leq 5$.

[a] *School of Chemical Sciences; The University of Auckland; 23 Symonds St; Auckland 1142, New Zealand.*
[b] *School of Biological Sciences; The University of Auckland; 3A Symonds St; Auckland 1142, New Zealand.*
[c] *Department of Wine and Food Science; The University of Adelaide; Wine Innovation Central; Adelaide SA 5064; Australia.*
† lisa.pilkington@auckland.ac.nz; d.barker@auckland.ac.nz; b.fedrizzi@auckland.ac.nz.
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Saccharomyces cerevisiae (*Sc*) yeast, which is used widely as a eukaryote model, contains known orthologues (*CYS4* and *CYS3*, respectively[11]) of the mammalian cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) genes, implicated in the synthesis of polysulfides.[8, 12, 13] Other putative orthologues of mammalian genes implicated in the sulfur pathway in yeast include *GLO1*,[14] *IRC7*[15] and *TUM1*.[16] It has also been proposed that *Sc* Tum1p may produce CysSSH from H_2S using the same mechanisms as mammalian 3-mercaptopyruvate sulfurtransferase (3MST).[17] Nonetheless, polysulfides have not yet been identified in yeast. Detection of these metabolites in yeast will advance our knowledge of the role of polysulfides and H_2S in signaling and cellular processes.

To confirm the presence of polysulfides in yeast, authentic samples were required for the analysis. However, previous syntheses of polysulfides ($R(S)_nH/R(S)_nR$ where R is GSH, Cys, and $2 \leq n \leq 5$) are limited, with few examples of their preparation. Of the hydropolysulfides, only the sodium salt of CysSSH has previously been prepared and isolated.[18] The lack of prior syntheses is largely due to the instability of hydropolysulfides, which degrade to the monosulfide species and elemental sulfur, with $T_{1/2}$<2 min in aqueous systems.[18] Additionally, the insolubility of Cys and GSH in most organic solvents render them difficult to work with when using reagents previously employed in syntheses of non-natural polysulfides. Generally protecting groups have been used to circumvent these issues.[19] Unfortunately, these groups are difficult to remove without affecting polysulfide moieties. The combination of these factors means that previous synthetic methods for generating hydropolysulfides were inapplicable to this work. While Cys and GSH hydropolysulfides are unstable, the oxidized polysulfides have greater stability. However, there are limited examples of previous syntheses of these substrates[8] and no examples of $CysS(S)_nCys$ and $GS(S)_nG$ with n > 2 selective synthesis. Due to the instability of hydropolysulfides, we derivatized these substrates using monobromobimane (mBB) to add a bimane fluorophore[20], effectively forming an oxidized polysulfide.

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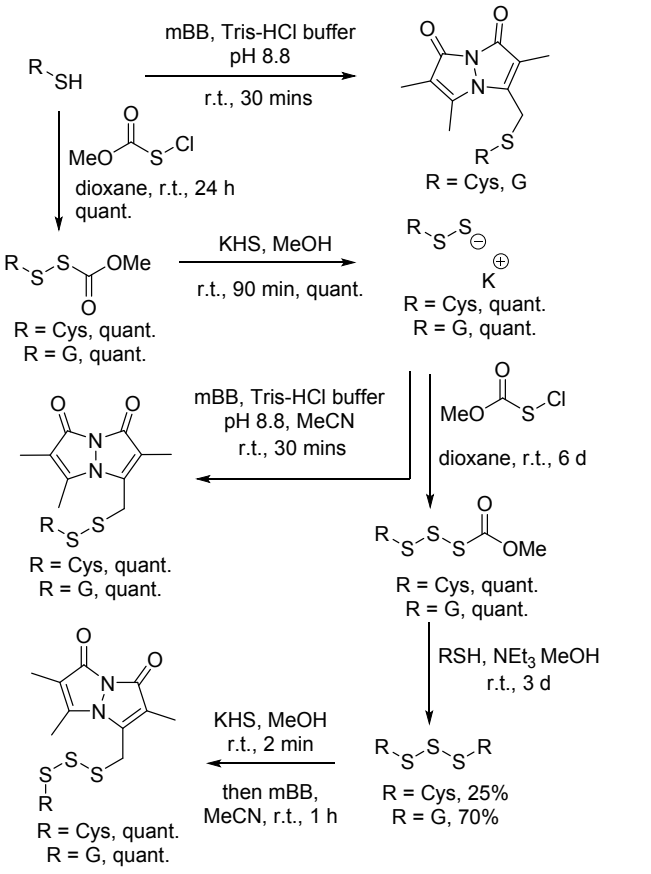
To start, Cys was reacted with methoxycarbonylsulfenyl chloride to provide the *O*-methyl carbono(dithioperoxoate) in quantitative yield, which was then reacted with potassium hydrosulfide (KHS), giving the desired hydropolysulfide, **CysSSK**, which was subsequently derivatized using the optimized method (SI) to give **CysSSBim** (Scheme 1, Fig. S2). Next, **CysSSK** was reacted with methoxycarbonylsulfenyl chloride, giving the trisulfane carboxylate, which was further reacted with KHS, followed by derivatization of the resultant hydrotrisulfide, giving **CysSSSBim** (Fig. S2). The trisulfane carboxylate was also reacted with Cys and Et_3N, providing **CysSSSCys**. This process was repeated with GSH, producing the corresponding GSH polysulfides, **GSSBim, GSSSBim** and **GSSSG** constituting the first selective synthesis of Cys and GSH oxidized and hydropolysulfides. Procedures adapted from the literature were then used to provide $CysS(S)_nCys$ and $GS(S)_nG$, with n = 2-5 and $Cys(S)_nBim$ and $G(S)_nBim$, where n = 1-4 (Schemes S2, S3 and Fig. S3, S4).[8]



Scheme 1. Iterative synthesis of oxidized polysulfides and hydropolysulfides of interest. Cys = cysteine, G = glutathione.

To identify the presence of polysulfides in *Sc* cells, and explore the role of yeast enzymes in their biosynthesis, a series of bioassays with single sulfur sources (sulfate (SO_4^{2-}), Cys (CysSH) and cystine (CysSSCys)) were performed.

The first bioassay employed fluorescent staining using the SSP4 probe which binds to sulfane sulfur,[8, 21-23] followed by visualization using fluorescence microscopy. Fig. S11A shows the absence of fluorescence or autofluorescence by *Sc* BY4743 cells in the negative control without SSP4 when supplied with Cys as the sole sulfur source. Fig. S11B shows the fluorescent staining of positive control samples with added $Cys(S)_nCys$, synthesized as described above. As the exposure time for Fig. S11A and S11B was the same, the brighter background fluorescence in Fig. S11B is likely to be from polysulfide that was not taken up by *Sc*. Fig. S11C-E show fluorescence via likely formation of polysulfides in *Sc* on three different sulfur sources, with staining not fully homogenous within the cells.

Furthermore, an *in-house* LC-MS/MS polysulfidomics approach, adapted from literature was employed.[8] Semi-quantitative analysis of different polysulfides produced intracellularly by *Sc* BY4743 and three single gene deletants (*Δcys3*, *Δcys4* and *Δtum1*), with known roles in the sulfur pathway, was carried out to begin unravelling how these compounds are formed.

CYS3, *CYS4* and *TUM1* were selected based on their orthology to mammalian CSE, CBS[11] and 3MST, respectively.[16] Retention times were determined by analyzing synthesized polysulfides. GSSSG and CySSSH were not present in *Sc*. The absence of GSSSG was unexpected based on recent results from Jastrzembski et al.[24] however, their experimental conditions were markedly different (S_0 as a sulfur source in a high sugar medium). Peak areas (normalized to account for differences in cell number, see Table 1) for detected compounds, across three sole sulfur sources and for BY4743 and the three mutants are represented in Fig. S12. This is the first study to show that hydropolysulfides accumulate in *Sc* and corroborates the viability of using *Sc* as a model organism for polysulfidomics.

Sulphate	GSH	GSSH	GSSG	CysSSH	CysSSSSH
BY4743	**46439**	**909**	**2654**	**ND**	**115**
BY4743 Δcys3					
BY4743 Δcys4					
BY4743 Δtum1					

Cysteine	GSH	GSSH	GSSG	CysSSH	CysSSSSH
BY4743	**16366**	**655**	**2064**	**ND**	**131**
BY4743 Δcys3					
BY4743 Δcys4					
BY4743 Δtum1					

Cystine	GSH	GSSH	GSSG	CysSSH	CysSSSSH
BY4743	**1023**	**361**	**88**	**ND**	**107**
BY4743 Δcys3					
BY4743 Δcys4					
BY4743 Δtum1					

Table 1: Z-Scores from the statistical comparison of the amount of polysulfide for SO_4^{2-} (top), Cys (middle) and cystine (bottom), for each gene-deletant compared to the reference (BY4743, peak value relative to cell density, two-sample t-test). Blue = greater levels of the polysulfide vs. the reference. Red = lower levels of the polysulfide vs. the reference. The intensity of color indicates statistical significance (no color (z-score < 1); light (1 < z-score < 2); medium (2 < z-score < 3); dark (z-score > 3)).

Examining the BY4743 reference strain, the most abundant species was GSH. *Sc* is known to produce reduced and oxidized GSH, at concentrations between 1-10 mM.[25, 26] SO_4^{2-} gave the largest peak areas for GSH, followed by Cys then cystine. GSH is a key precursor for formation of polysulfide species.[8] Peak areas for GSSH and GSSG followed a similar pattern to GSH. All sulfur sources were able to induce the formation of GSSH. There was also a step-wise decrease from GSH to GSSH and GSSG to GSSSG

for all sulfur sources, which is consistent with GSH being in equilibrium with GSSG, but with GSH:GSSG concentrations at a ratio of 50:1, due to the reducing cellular environment.[27] No CysSSH or CysSSSH was detected in BY4743. The absence of CysSSH was unexpected, particularly with cystine as the sole sulfur source, since it is known that CysSSH is formed from cystine.[8] The presence of CysSSH may be highest during the exponential phase of *Sc* growth and once cells reach stationary phase, as in our study, CysSSH may already be depleted to form GSSH. Another notable observation includes the presence of CysSSSSH in BY4743, with no difference between sulfur sources. Yeast is clearly able to make GSH-derived hydropolysulfides and oxidized polysulfides, as well as Cys-derived hydropolysulfides, and the sulfur source used to grow the yeast alters polysulfide content.

To identify the potential role of yeast genes in polysulfide formation, we compared results obtained for BY4743 to three single gene deletants. Correlation profiles show that SO_4^{2-} produces more polysulfides overall and interrelationships between genes and polysulfides are stronger and more statistically significant than for Cys or cystine. The presence of Tum1p and Cys3p has a negative effect on the amounts of all polysulfides observed, while the presence of Cys4p has a varying effect, depending on the polysulfide. Alternatively, with Cys as the sulfur source, all significant differences in the levels between BY4743 and the gene deletants were positive. These complex interrelationships were analysed further using heatmaps (Fig. S13) and networks (Fig. 2). The networks demonstrate the positive relationship between GSH and GSSH and GSSG for all sulfur sources, but differing relationship between CysSSSSH and these GSH species depending on sulfur source. For SO_4^{2-} and cystine, there is a negative relationship; as levels of GSH/GSSG/GSSH increase, the level of CysSSSSH decreases, and vice versa, whereas when Cys is the sulfur source, there is a positive correlation. There is also a positive correlation between CysSSSSH and CysSSH for the sulfur sources producing both of these compounds.

In terms of specific gene effects on polysulfide production, *Δcys4* (SO_4^{2-} or cystine as sole sulfur source) and *Δtum1* (SO_4^{2-} or Cys) were the most different compared to BY4743. We expected that the deletion of *Δcys3* (orthologue of CSE[28]) would result in a diversion towards the production of polysulfides derived from Cys via Tum1p.[17,11] This would also reduce the formation of specific polysulfides species formed from the H_2S produced via Cys degradation. For *Δcys3*, concentrations of GSH, GSSH and GSSG were slightly higher than in BY4743, particularly with Cys and cystine as the sulfur source. This appears contrary to results in mammals showing that CSE has higher *in vitro* activity than CBS for polysulfide formation.[8] It is possible that the slight increase in GSSH and GSSG can be explained by the action of Tum1p on Cys, resulting in formation of Cys polysulfides, which are precursors to GSH-derived polysulfides.[8, 16] Subsequent to this observation, there was an increase in CysSSH with Cys and cystine as sole sulfur sources, but not for SO_4^{2-}. It is possible that more Cys or cystine is diverted to CysSSH in the *Δcys3* mutant and perhaps Cys3p is required later in the pathway. Based on

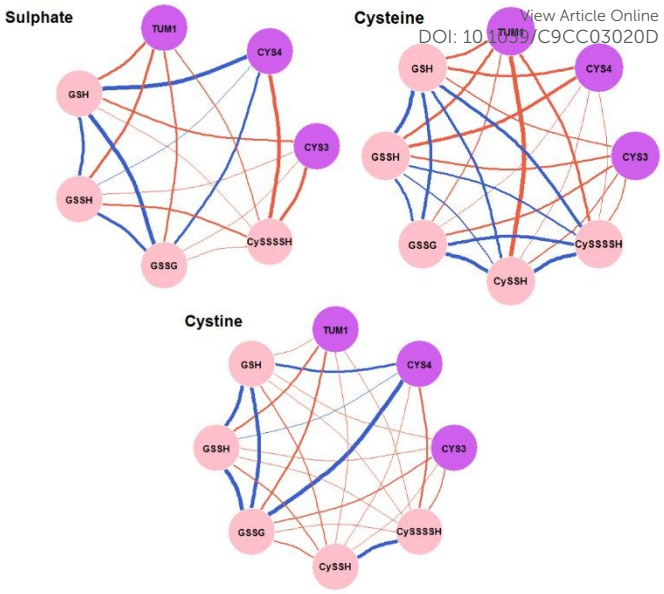


Figure 2: Networks exploring the interrelationships between gene and polysulfides: SO_4^{2-} as the sulfur source (top left), Cys as the sulfur source (top right) and cystine as the sulfur source (bottom). Nodes are colored to represent gene (purple) or polysulfide species (pink). Edges between gene and polysulfide are colored according to the apparent effect of the gene on the occurrence of polysulfide (*i.e.* blue indicates a positive effect, red indicates a negative effect) and the weighting signifying the strength of the effect. Edges between polysulfides are colored according to the correlation (*i.e.* blue indicates a positive correlation, red a negative correlation), with the line width signifying the strength of the correlation.

previous observations,[29] where excess polysulfides, including CysSSH, were accumulated under conditions of oxidative stress, it is possible that there is a loss of control over polysulfide regulation in a *Δcys3* mutant compared to BY4743 suggesting that under normal conditions, BY4743 does not accumulate these species. This idea is supported by the networks which demonstrate that presence of *CYS3* negatively impacts the occurrance of all polysulfide species across all sulfur sources. Another hypothesis proposed is that the Irc7p β-lyase enzyme in yeast may act on cystine to produce CysSSH,[15] effectively functioning like mammalian CBS and CSE and another source of the GSH-derived polysulfides in *Sc*.

Cys4p is hypothesized to act in a similar fashion to Cys3p. [8] From our results it appears that there are key differences between the relative amounts of polysulfides produced with different sulfur sources by Cys3p and Cys4p. For *Δcys4*, the concentration of GSH was significantly lower for SO_4^{2-} than Cys, while this was not mirrored in the *Δcys3* mutant. The same pattern was seen for GSSH and GSSG. CysSSH was present in *Δcys4* mutant cells when grown on Cys and cystine, similar to the *Δcys3* mutant. As suggested for *Δcys3*, the *Δcys4* mutant may have disrupted utilization or conversion of CysSSH. Differences in conversion efficiency and substrate specificity of these enzymes to form CysSSH could explain why peak areas for CysSSH are higher in *Δcys3* compared to *Δcys4* when grown on Cys, but vice versa on cystine. The fact that *Δcys4* was defective in growth (Table S3), may also affect the profile of polysulfides produced.

Deletion of *TUM1*[30, 31] has recently been shown to result in a dramatic reduction in H_2S produced from Cys, potentially

forming Cys persulfides from H_2S and behaving similarly to mammalian 3MST.[16] Based on this information, deletion of *TUM1* was expected to result in a reduction of Cys persulfides derived from Cys. However, the presence of *TUM1*, not its absence, appears to be correlated with a decrease in all polysulfide species. For example, the *Δtum1* mutant displayed larger peak areas for GSSH and GSSG in SO_4^{2-} and Cys compared to BY4743. This could be due to increased Cys leading to increased GSH, hence the ability to form more GSH-derived polysulfides. It is also noteworthy that CysSSH was produced only from Cys in *Δtum1* cells. Therefore, cells can still produce CysSSH from Cys without a functional Tum1p enzyme, meaning there could be other yeast genes capable of producing CysSSH from Cys.

This study has shown for the first time the occurrence and biosynthesis of polysulfides in yeast. Using a novel synthetic approach to prepare specific polysulfides in combination with LC-MS/MS methodology we have shown the viability of using yeast as a model system for complex semi-quantitative polysulfidomics. Furthermore, we have provided evidence that altering the sulfur source for yeast growth alters the polysulfide content and started to unravel the roles of *Sc* genes in polysulfide biosynthesis.

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Conflicts of interest

There are no conflicts to declare.

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