An evolutionary approach uncovers a diverse response of tRNA 2-thiolation to elevated temperatures in yeast

FIONA ALINGS,1,5 L. PETER SARIN,1,5 CHRISTIAN FUFEZAN,2 HANNES C.A. DREXLER,3 and SEBASTIAN A. LEIDEL1,4

1RNA Biology Laboratory, Max Planck Institute for Molecular Biomedicine, 48149 Münster, Germany
2Institute of Plant Biology and Biotechnology, University of Münster, 48143 Münster, Germany
3Bioanalytical Mass Spectrometry Unit, Max Planck Institute for Molecular Biomedicine, 48149 Münster, Germany
4Faculty of Medicine, University of Münster, 48149 Münster, Germany

ABSTRACT
Chemical modifications of transfer RNA (tRNA) molecules are evolutionarily well conserved and critical for translation and tRNA structure. Little is known how these nucleoside modifications respond to physiological stress. Using mass spectrometry and complementary methods, we defined tRNA modification levels in six yeast species in response to elevated temperatures. We show that 2-thiolation of uridine at position 34 (s²U₃⁴) is impaired at temperatures exceeding 30°C in the commonly used Saccharomyces cerevisiae laboratory strains S288C and W303, and in Saccharomyces bayanus. Upon stress relief, thiolation levels recover and we find no evidence that modified tRNA or s²U₃⁴ nucleosides are actively removed. Our results suggest that loss of 2-thiolation follows accumulation of newly synthesized tRNA that lack s²U₃⁴ modification due to temperature sensitivity of the URM1 pathway in S. cerevisiae and S. bayanus. Furthermore, our analysis of the tRNA modification pattern in selected yeast species revealed two alternative phenotypes. Most strains moderately increase their tRNA modification levels in response to heat, possibly constituting a common adaptation to high temperatures. However, an overall reduction of nucleoside modifications was observed exclusively in S288C. This surprising finding emphasizes the importance of studies that utilize the power of evolutionary biology, and highlights the need for future systematic studies on tRNA modifications in additional model organisms.

Keywords: yeast; tRNA modification; URM1; temperature stress; evolution

INTRODUCTION
Transfer RNA (tRNA) is critical for translation by linking the codon of the messenger RNA (mRNA) to the appropriate amino acid (Crick et al. 1957). Interestingly, all tRNA molecules feature post-transcriptional chemical modifications, some of which are highly conserved throughout all domains of life (Grosjean 2009; Grosjean et al. 2014). To date, ~110 RNA modifications have been reported, and among the 42 tRNA species found in yeast, 25 modifications occur at 36 individual nucleotide positions (Phizicky and Hopper 2010). The greatest diversity of modified nucleotides is found at the anticodon loop positions 34 and 37—two nucleotides that are critical for decoding and maintenance of the translational reading frame (Helm and Alfonzo 2014). Furthermore, some modifications at the tRNA core affect the stability of specific tRNA species, whereas only minor effects are seen on other tRNAs (Phizicky and Alfonzo 2010).

Little is known about the regulation of tRNA modifications and how they respond to external factors. Certain nucleoside methylations were reported to increase upon chemical stress, which might indicate a need for tighter structural rigidity (Chan et al. 2010, 2012). Moreover, it has been proposed that tRNA modification changes might affect translation in response to stress (Begley et al. 2007; Chan et al. 2010, 2012; Bauer et al. 2012; Laxman et al. 2013). An interesting example is uridine at position 34 (U₃⁴), which pairs with the wobble base of the codon. In isoacceptors tE₃⁴UU, tK₃⁴UU, and tQ₃⁴UG, U₃⁴ is modified to 5-methoxycarbonylmethyl-2-thiouridine (mcm⁵s²U). The mcm⁵s²U modification is generated through the concerted action of the elongator (ELP) pathway, which is required for formation of the mcm⁵ side chain, and the URM1 pathway that is essential for 2-thiolation (s²) (Huang et al. 2005, 2008; Björk et al. 2007; Nakai et al. 2008; Schlieker et al. 2008; Leidel et al. 2009; Noma et al. 2009). The physiological role of mcm⁵s²U is not well established, although it is thought to

5These authors contributed equally to this work.
Corresponding author: sebastian.leidel@mpi-muenster.mpg.de

© 2015 Alings et al. This article, published in RNA, is available under a Creative Commons License (Attribution-NonCommercial 4.0 International), as described at http://creativecommons.org/licenses/by-nc/4.0/.
stabilize codon–anticodon interactions (Grosjean 2009; Rodríguez-Hernández et al. 2013), and yeast strains lacking U\textsubscript{34} modifications are sensitive to high temperatures and chemical stress (Esberg et al. 2006; Leidel et al. 2009).

Studies that connect tRNA modifications to stress generally apply harsh chemical treatments that are often incompatible with cellular viability. This complicates the evaluation of such a relationship and it is therefore desirable to analyze physiological stress conditions. Moderate temperature stress constitutes a key example of such a physiologically relevant experimental setup. The effect of elevated temperatures is well established on the transcriptional level in Saccharomyces cerevisiae, where >1000 genes respond to temperature shifts (Gach et al. 2000). However, little attention has been paid to genes that are required for tRNA modification. Furthermore, temperature-induced changes in the tRNA modification landscape have not been extensively analyzed in eukaryotes, although studies have been made in bacteria and archaea (Agris et al. 1973; Watanabe et al. 1976; Kumagai et al. 1980; Kowalak et al. 1994). This is surprising, as growth at high temperature is associated with pathogenicity of yeast (McCusker et al. 1994), and a detailed study of tRNA modifications could potentially reveal mechanisms that facilitate temperature tolerance.

Finally, the majority of studies in yeast utilize a few common laboratory strains of S. cerevisiae. These strains have been domesticated and evolved in an environment that differs markedly from their natural habitat (Liti et al. 2009). Therefore, it is an open question whether such strains might respond to environmental stress differently than yeasts that have not evolved in the laboratory for thousands of generations.

To elucidate how cells respond to stress, we set out to analyze how high temperatures affect tRNA modifications in yeast. To this end, we quantitatively characterized nucleoside modification levels in six yeast species—S. cerevisiae, Saccharomyces bayanus, Saccharomyces mikatae, Saccharomyces paradoxus, Candida glabrata, and Schizosaccharomyces pombe—at ambient and elevated temperatures. We used reversed-phase ultraperformance liquid chromatography–mass spectrometry (RP-UPLC–MS), affinity gel electrophoresis, and Northern blots. This allowed us to establish the first comparative inventory of tRNA modifications in several yeast species. To our knowledge, this is the first comprehensive evolutionary analysis of how tRNA modifications respond to temperature stress. We found that high temperatures induced profound changes in the tRNA modification landscape. Surprisingly, all strains but one responded by globally up-regulating modification levels. We observed the opposite effect only in S288C. When specifically analyzing mcm\textsuperscript{3}U\textsubscript{34}, we detected a significant decrease in 2-thiolation at high temperature in S. cerevisiae and S. bayanus. This reduction is reversible and recovers fully upon removal of the stress. Using polymerase inhibitors and a temperature-sensitive mutant strain of RNA polymerase III, we found no evidence that thiolation is actively down-regulated. In contrast, our results suggest that the URM1 pathway is inactive at high temperatures, and that loss of thiolation occurs as a result of diluting modified tRNA by newly transcribed unmodified species. Moreover, mcm3\textsuperscript{3}U\textsubscript{34} modification levels are not negatively affected. Taken together, these findings suggest that the URM1 pathway is temperature sensitive in S. cerevisiae and S. bayanus, potentially reflecting a domestication event.

RESULTS

The S. cerevisiae S288C and W303 strains are capable of growth at elevated temperatures

We sought to quantitatively characterize tRNA modification landscapes of yeast in response to a relevant physiological stress. In most natural environments yeasts experience high temperatures, making it a very appropriate experimental paradigm. Furthermore, some tRNA modification mutants confer temperature sensitivity, implying a connection between temperature stress and tRNA modification (Esberg et al. 2006; Phizicky and Hopper 2010). We first determined the upper temperature limit of two commonly used laboratory strains—S288C and W303—by serial dilution spotting at temperatures ranging between 25°C and 43°C (Fig. 1). Both colony morphology and growth were normal at temperatures of up to 37°C. Interestingly, S288C is capable of growing at 39°C, whereas W303 is significantly impaired at this temperature. Neither of the strains was able to grow at temperatures exceeding 40°C (Fig. 1).

Mass spectrometry analysis of tRNA modification levels reveals unexpected variation

To quantitatively characterize tRNA modifications, we grew yeast at different temperatures in liquid culture and isolated their tRNA. The total tRNA was enzymatically converted into mononucleosides, which we analyzed by RP-UPLC–MS (Fig. 2A). Overlapping nucleosides were differentiated based on at least two of the following criteria: the isotopologue profile, the reported mass/charge (m/z) ratio, or the expected mass/charge (m/z) ratio, or the expected
neutral loss—i.e., the mass change induced by dissociation of the ribosyl moiety from the nucleobase during in-source fragmentation. If the nucleobase is methylated, loss of the ribose yields a mass change of 132.04, whereas methylation of the ribose leads to a neutral loss of 146.06 (Fig. 2B). In addition, lack of neutral loss serves as a distinguishing feature to discriminate pseudouridine (Ψ) from uridine (Dudley et al. 2005). To compare the relative change in tRNA modification levels as a function of temperature, we adapted pyQms in a newly developed pipeline system for the analysis of nucleoside modifications. PyQms is a Python module for high-throughput quantification of MS experiments (Barth et al. 2014). Briefly, the area under the curve was determined for each modification from the MS1 spectra and normalized against adenosine. The normalized values were then compared with the respective value at the reference temperature (30°C) to reveal relative changes in modification levels. The feasibility of this approach, as well as the technical reproducibility and sensitivity of the LC–MS instrument, was validated by a set of 18 chemically synthesized nucleoside modifications (Supplemental Fig. S1A).

Manual analysis of the MS data for S288C at 30°C revealed 25 chemical modifications, of which pyQms reproducibly detected and quantified 21 (Fig. 2C; Supplemental Fig. S1B). We observed a clear temperature-dependent decrease in modification levels for S288C. At 25°C and 34°C, most nucleoside modifications are 1.2- to 1.8-fold less abundant than at 30°C, whereas dihydrouridine (D) and 7-methylguanosine (m7G) remain largely unaffected (Fig. 2C; Supplemental Table S1). However, a significant reduction occurs at 37°C and 39°C where all modifications are on average approximately fourfold down-regulated, apart from 2′-O-methyladenosine (Am) that remains largely unaltered (Fig. 2C; Supplemental Table S1). Interestingly, at elevated temperatures Ψ levels decrease noticeably (~10-fold reduction at 39°C), whereas modifications associated with the structural stability of tRNA molecules, such as 1-methylguanosine (m1G) and 2-methylguanosine (m2G), are less affected (~3.0-fold). Indeed, the relative abundance of m1G and m2G at 37°C and 39°C increases by ~38% and ~12%, respectively (Fig. 2C; Supplemental Table S1). In contrast, wobble position modifications, including 2′-O-methylcytidine (Cm) and 2′-O-methylguanosine (Gm) decrease strongly (Fig. 2C).

The W303 and S288C strains are evolutionarily closely related (Liti et al. 2009). Surprisingly, their modification landscape differs markedly (Fig. 2C). A general reduction in modification levels following temperature stress could not be seen for W303. Most nucleoside modifications remain at relatively high levels and rise, as temperature increases (Fig. 2C). The 1-methyladenosine (m1A) and Ψ modifications increase 1.4- and 1.9-fold, respectively, at 39°C (Supplemental Table S1). The same trend is observed for many other modifications, with m2G, Cm, and m3G being notable exceptions (Fig. 2C). In particular, m2G decreases to nearly nondetectable levels at 37°C and 39°C. Furthermore, when
comparing the overall modification landscape of W303 to S288C, it appears that W303 is hypomodified at ambient temperatures but becomes hypermodified at 39°C (W303: ∼1.4-fold reduction at 25°C, ∼1.5-fold increase at 39°C; S288C: ∼1.2-fold reduction versus ∼0.4-fold reduction). This shows that although S288C and W303 may have a similar genotype, their tRNA modification phenotypes differ substantially.

### 2-Thiolation of tRNA decreases as a response to temperature stress

This perplexing difference in tRNA modifications observed in our MS analysis prompted us to perform an in-depth analysis of one specific modification. We chose mcm^5s^2U^34 for several reasons. First, we did not detect this modification by MS, but it can be accurately quantified by ([N-acryloylaminophenyl]mercuric chloride (APM)-affinity gel electrophoresis and Northern blotting with radiolabeled probes. In this assay, thiolated tRNAs are specifically retarded in a polyacrylamide gel (Igloi 1988). Second, mcm^5s^2U^34 only occurs on three cytoplasmic tRNAs—tKUUU, tQ^UUU^ and tQ^UG^—allowing us to quantify all modification targets in parallel. Third, this wobble uridine modification has been implicated in translational control (Grosjean 2009; Rodriguez-Hernandez et al. 2013).

Strikingly, we found a significant reduction in mcm^5s^2U^34 levels at elevated temperatures (Fig. 3). For S288C, ∼60% of tK^UUU^ is modified at 25°C and 30°C, whereas modification levels drop below 20% at 39°C (Fig. 3A). A slight decrease to ∼50% is seen at 34°C, suggesting that this temperature constitutes the upper boundary for the thermal adaptation of the strain. A similar trend is observed for tE^UUC^ as well, albeit the overall 2-thiolation levels are slightly lower at all temperatures. Interestingly, tQ^UUU^ shows even lower 2-thiolation levels (∼35%) at 25°C–34°C, and an almost complete loss of 2-thiolation at 37°C and 39°C (Fig. 3A). This indicates that the specificity or affinity of the enzymes toward the individual isoacceptors differ substantially in S288C. In W303, 2-thiolation levels for all three isoacceptors are more similar, suggesting that formation of the mcm^5s^2U^34 modification proceeds with a lower isoacceptor preference (Fig. 3B). In contrast to most other modifications and as observed in S288C, tRNA thiolation levels clearly decrease at elevated temperatures in W303.

We independently verified our results for the S288C samples by UPLC. As seen in the chromatogram overlay, the intensity of the mcm^5s^2U^34 signal decreased as temperature increased (∼0.61% of the total signal at 30°C, ∼0.28% at 34°C, ∼0.071% at 37°C, and ∼0.021% at 39°C) (Fig. 3C). This analysis confirmed that 2-thiolation levels are reduced at higher temperatures, although it did not provide information regarding possible isoacceptor preferences.

2-Thiolation levels are reduced in ELP-complex deficient strains (Björk et al. 2007; Leidel et al. 2009). Such a deficiency could account for the reduction in 2-thiolation that we observed. However, careful analysis of MS data speaks against this. In W303 cells, mcm^5U^14 levels increase ∼1.2-fold at 34°C to ∼3.8-fold at 39°C, whereas mcm^5s^2U^14 levels decrease approximately threefold to fivefold depending on the isoacceptor (Supplemental Table S1; Figs. 2C, 3B). This suggests that the ELP pathway remains active. In S288C, the picture is more complicated due to the overall reduction of most modifications at high temperatures. However, mcm^5U^14 levels can act as a proxy for ELP pathway activity that is independent of s^2 levels (Björk et al. 2007). The ratio of mcm^5U^14 to mcm^5s^2U^14 increases in S288C from ∼1.3-fold at 34°C to ∼2.5-fold at 37°C, and in W303 from ∼1.4-fold at 39°C.
34°C to ~1.8-fold at 37°C and 39°C, respectively. This indicates that the ELP pathway is active and relative increases in mcm5s2U34 levels are observed due to lack of 2-thiolation. Thus, the observed loss of mcm5s2U34 modification at high temperatures is not due to a defect in the ELP pathway, but rather due to temperature sensitivity of one or more components in the URM1 pathway.

2-Thiolation recovers upon removal of temperature stress

To the best of our knowledge, temperature sensitivity of a cytoplasmic U34 modification has not been described before. Therefore, we wanted to better understand the kinetics of this inactivation and to test whether 2-thiolation levels can recover. To address this question, we maintained S288C cells in logarithmic growth at 37°C for 7 h, at which point the cultures were split into two. One culture was kept at 37°C and the other shifted to 30°C, and growth was continued for additional 7 h (Fig. 4A). We found that the mcm5s2U34 modification levels decreased at 37°C in an exponential manner, reaching the half point after ~3 h (Fig. 4B; Supplemental Fig. S2). However, in cells that were shifted to 30°C, 2-thiolation levels recovered within a few hours. This shows that cells maintain the ability to recover.

Inhibition of tRNA synthesis stalls 2-thiolation at elevated temperatures

We have demonstrated that loss of mcm5s2U34 modification is reversible in wild-type yeast. However, different mechanisms could underlie the loss of 2-thiolation at higher temperatures: 2-thiolated tRNA molecules might be actively degraded, the modification itself might be actively removed, or newly transcribed tRNA might not acquire the modification at 37°C, thereby diluting the modified tRNA.

To address this question, we decided to inhibit RNA synthesis. First, we used ML-60218, an indazolo-sulfonamide compound that specifically inhibits RNA polymerase III (Wu et al. 2003). ML-60218 does not completely inhibit tRNA synthesis, but a leakiness of 20%–30% is expected (Iwasaki et al. 2010). This hypomorphic phenotype allowed us to reduce tRNA synthesis while only slightly reducing growth rates during the first hours of the experiment (Fig. 5A, top panel). Importantly, the loss of tRNA modification occurred slower relative to the situation without the inhibitor (Figs. 4B, 5B; Supplemental Figs. S2, S3A). This suggests that tRNA synthesis rather than active removal of thiolated tRNA or thiolated nucleosides affect thiolation levels at high temperatures.

To verify this hypothesis, we used thiolutin to completely block transcription of RNA polymerases I–III (Tipper 1973). Addition of thiolutin led to cessation of growth within 1 h (Fig. 5A, middle panel). Similarly, the mcm5s2U34 modification levels remained constant throughout the experiment (Fig. 5C; Supplemental Fig. S3B). To exclude that this is an indirect effect caused by inactivation of RNA polymerases I and II, we utilized S. cerevisiae rpo31-698, a temperature-sensitive mutant strain of RNA polymerase III (Li et al. 2011). When shifted to the restrictive temperature at 37°C, growth slowed down (as seen for ML-60218) (Fig. 5A) and 2-thiolation levels remained constant following an initial decrease during the first hour after shift (Fig. 5D; Supplemental Fig. S3C). These experiments suggest that the tRNA isoacceptors and the modification per se remain stable and are not actively turned over. It is unlikely that addition of the inhibitors directly affect 2-thiolation levels, as we observed no change in mcm5s2U34 levels in cells grown at 30°C in the presence of ML-60218 or thiolutin (Supplemental Fig. S4A,B). Furthermore, cells grown at 37°C and shifted to 30°C upon addition of thiolutin did not increase 2-thiolation levels (Supplemental Fig. S4C). Taken together, our experiments suggest that tRNA synthesized before exposure to heat retain their modifications whereas tRNA synthesized at high temperatures is not 2-thiolated.

Temperature tolerance and 2-thiolation vary markedly in other yeast species

As we found reduced s2U34 levels at elevated temperature in S. cerevisiae, we...
wondered whether this trait is evolutionarily conserved and constitutes a common adaptation of yeast to high temperature. To this end, we analyzed representative strains of five evolutionarily divergent yeast species; *S. bayanus*, *S. mikatae*, *S. paradoxus*, *C. glabrata*, and *S. pombe* (Fig. 6A), and performed serial dilution spottings at the same temperature range as for *S. cerevisiae*. In *S. mikatae*, *S. paradoxus*, and *S. pombe*, we did not detect obvious changes in 2-thiolation levels in response to heat (Fig. 7B, C, E). However, in *C. glabrata*, which is a pathogenic yeast species capable of growth at high temperature, 2-thiolation levels rose slightly in response to increased temperature (Fig. 7D). Thus, loss of 2-thiolation does not constitute a conserved response to high temperatures in yeast.

Furthermore, MS analysis of these representative yeast species revealed tRNA modification landscapes that share remarkably similar features (Fig. 8; Supplemental Fig. S5). In contrast to S288C, all other strains share the trend of up-regulating modification levels at higher temperatures (Figs. 2C, 8). In particular, *S. bayanus* is very similar to W303 as many modifications respond comparably with increased temperatures (Figs. 2C, 8). *Candida glabrata* surpasses its modification levels at 30°C by an average of ∼12% at 39°C–43°C. With the exception of S288C, Ψ modification levels increase ∼1.2- to 2.0-fold at high temperatures in all other strains analyzed (Fig. 8; Supplemental Table S1). Methylations associated with the structural stability of the tRNA molecule, such as m5C and m1G, show clear temperature dependence in all species and become more prevalent as temperature increases (Fig. 8). More divergence is observed for position 34 modifications, as mc5U and Am remain stable throughout all temperatures in all species, and Cm decreases significantly in *S. mikatae* and *C. glabrata* but not in *S. pombe* (Fig. 8; Supplemental Table S1). In addition, m7G is detected at slightly higher intensity only at ambient temperature in *S. bayanus* and *S. pombe*. This surprising similarity between species implies that neither evolutionary distance nor natural environments have forced a radiation of modification phenotypes.

**DISCUSSION**

We conducted a comprehensive evolutionary study to quantitatively characterize the effects of temperature stress on tRNA modification levels in yeast. Importantly, we
established that elevated temperatures lead to a reduction of the mcm5 s\(^2\)U\(_{34}\) level in the commonly used \(S\). \(cerevisiae\) laboratory strains S288C and W303. However, this reduction is reversible, as 2-thiolation levels return to normal within a few hours after relief of the stress. This is an intriguing finding, as modification of U\(_{34}\) in t\(E_{\text{UUC}}\) has been proposed to dynamically regulate the translation of key damage response proteins (Begley et al. 2007). A decrease of s\(^2\)U\(_{34}\) might similarly redirect the translational program of cells at high temperature by disfavoring recognition of the cognate codons for t\(E_{\text{UUC}}\), t\(K_{\text{UUU}}\), and t\(Q_{\text{UUG}}\). However, it is unlikely that loss of 2-thiolation in response to high temperatures might be an active regulatory process, which is exploited by the cell. First, the decrease in 2-thiolation levels correlates with cellular growth rates, suggesting that newly synthesized, unmodified tRNA dilutes the pool of modified tRNA. Similarly, recovery of thiolation upon shift to ambient temperatures is slow compared with the immediate response that follows environmental stress (Gasch et al. 2000). Thus, a translationally regulated response would be slow and follow the transcriptional rewiring of the cell rather than preceding it. Second, reducing or blocking the synthesis of nascent tRNA slows down or prevents loss of 2-thiolation. If s\(^2\)U\(_{34}\) would be actively degraded, it would likely be uncoupled from tRNA synthesis. Third, loss of U\(_{34}\) modification is likely to affect the translation of a large number of proteins, thereby causing cellular stress, which is corroborated by the up-regulation of Gcn4p upon U\(_{34}\) hypomodification (Zinshteyn and Gilbert 2013). Therefore, it is unlikely that \(S\). \(cerevisiae\) would gain fitness benefits for growth at high temperatures by actively reducing 2-thiolation. Finally, our analysis of other yeasts at high temperature reveals that s\(^2\)U\(_{34}\) levels are reduced only in \(S\). \(cerevisiae\) and \(S\). \(bayanus\). In contrast, their close relatives, as well as \(S\). \(pombe\), which constitutes the phylogenetic outgroup in our study, do not exhibit the same phenotype. Thus, a decrease of 2-thiolation in response to high temperatures is unlikely to constitute an ancient cellular program. Taken together, our results suggest that the reduction of s\(^2\)U\(_{34}\) levels at high temperatures is likely not an active regulatory process used to dynamically rewire the translational program of the cells. Rather, it may reflect the temperature sensitivity of one or more components of the \(URM1\) pathway.

It is puzzling that loss of 2-thiolation may have been independently acquired in \(S\). \(cerevisiae\) and \(S\). \(bayanus\). However, both were originally used in fermentation. It is possible that constant growth at ambient temperatures in rich media selected for modifications of cellular metabolism, which decrease fitness of yeast in its natural environment. Therefore, it will be interesting to extend the analysis to yeast strains from different ecological niches, and to test whether loss of 2-thiolation at high temperature is a general behavior of \(S\). \(cerevisiae\).

Importantly, our analysis of 2-thiolation reveals enzymatic features of the \(URM1\) pathway. We found that isoacceptors are not fully modified and that relative 2-thiolation levels generally follow the order t\(K_{\text{UUU}}\) > t\(E_{\text{UUC}}\) > t\(Q_{\text{UUG}}\). This

---

**FIGURE 6.** Evolutionary divergent yeasts have different temperature preferences. (A) Phylogenetic relationship and evolutionary distance of all yeast species included in this study. (Data adapted from Rhind et al. 2011.) (B) Serial dilutions (1:5) of five yeast species were spotted onto rich growth medium (YPD or YES) plates and incubated for 3 d at the indicated temperatures.
raises the question, whether functional constraints limit the pathway to fully modify all tRNA molecules or whether some isoacceptors are less important (Phizicky and Alfonzo 2010). The latter is supported by tRNA overexpression experiments in S. cerevisiae and S. pombe, where overexpression of tKUUU and tQUG, but not tEUUC, leads to the rescue of different stress sensitivities in mcm^s^U^34_4^ deficient strains (Esberg et al. 2006; Björk et al. 2007; Leidel et al. 2009; Fernández-Vázquez et al. 2013). Furthermore, 2-thiolation is not temperature sensitive in all yeasts. Thus, it is feasible to complement mutants in S. cerevisiae with the genes of other species that form s^U^34 at high temperature to identify the genes that cause temperature sensitivity of 2-thiolation.

We sought to define the tRNA modification landscapes in a range of yeast species in response to stress. By choosing representative strains of S. cerevisiae, S. bayanus, S. mikatae, S. paradoxus, and C. glabrata, we analyzed several prominent models in the Saccharomyces clade, including one pathogenic strain. Furthermore, S. pombe is separated from the other species due to a whole-genome duplication event (Vivancos et al. 2006). Surprisingly, all strains except S288C showed a fairly similar pattern—most tRNA modifications increase modestly if temperature rises >30°C. This may either reflect a coordinated response to temperature stress or a passive increase in pathway activities due to more favorable reaction conditions at higher temperatures. Our global analysis also did not address whether all tRNA modifications occur at relevant positions. Theoretically, the increase could reflect unspecific modifications through decreased selectivity toward the natural modification targets. However, examples in archaea show that tRNA modifications are more abundant at high temperature (Noon et al. 2003; Shigi et al. 2006). Similar findings have not been reported in a eukaryotic system. When looking at the modification landscapes more closely we find that Ψ, which is one of the most abundant nucleoside modifications, remains prominent at elevated temperatures in all strains analyzed, apart from S. cerevisiae S288C where it decreases. Clearly, methylations implicated in structural stability, such as m^5^C and m^1^G, become more prevalent at high temperatures, emphasizing a need for tighter conformational control of the tRNA molecule. Indeed, these observations support the notion that high temperature warrants increased tRNA modification levels also in eukaryotic cells.

Upon comparison to all other strains, it is surprising that the commonly used laboratory strain S288C reacts in such a divergent manner to increasing temperatures, especially as the response is so different from the closely related strain W303. It is important to keep this in mind. Even though S288C offers numerous experimental advantages, we need to be cautious when basing our conclusions on results obtained in a single wild-type strain. The selective pressures in the laboratory are unusual, and not every conclusion may stand the test in a natural environment. This emphasizes the need to perform more studies that make use of the great power of the evolutionary biology of yeasts.

**FIGURE 7.** 2-Thiolation levels are not affected by temperature in most other yeast species. Representative autoradiogram images of tRNA extracted at different growth temperatures from (A) S. bayanus, (B) S. mikatae, (C) S. paradoxus, (D) C. glabrata, and (E) S. pombe following APM-affinity gel electrophoresis and Northern blot with the tKUUU probe. 2-Thiolation levels were quantified by image densitometry (n = 3, apart from samples grown at 25°C where n = 2). Asterisks (*) show unspecific binding of the probe.
were collected at the indicated time points for tRNA isolation. At 7 h post-inoculation, the cultures were split into two, one being rhythmic growth by regular dilution with fresh prewarmed medium. Inoculated cultures at 37°C. The cultures were kept at loga-

This step was repeated twice. Subsequently, the total RNA contain-

FIGURE 8. tRNA modification landscapes for divergent yeast species show surprising similarity. Heat map of the relative change in nucleoside modification levels compared with 30°C in S. bayanus, S. mikatae, S. paradoxus, C. glabrata, and S. pombe using pyQms. The scale bar shows a 10-fold increase in modification levels as white, no change as pale orange, and a 31.6-fold decrease as black. Modifications that yield a weak MS signal are labeled in gray.

MATERIALS AND METHODS

Yeast strains and growth conditions

Haploid mating type a strains of S. cerevisiae and S. pombe were used and diploid strains of S. bayanus, S. mikatae, S. paradoxus, and C. glabrata. Refer to Supplemental Table S2 for further information about these strains.

All experiments were conducted in rich growth medium (YPD; Formedium), except for S. pombe, which was grown in YES. The solid growth media were supplemented with 2% agar. Overnight (o/n) starter cultures were grown at 30°C, 200 rpm. The starter culture was inoculated into prewarmed medium to yield an OD600 = 0.2, grown with 50 µg/mL N1-[3-(5-chloro-3-methylbenzo[b]thiophen-2-yl)-1-methyl-1H-pyrazol-5-yl]-2-chlorobenzene-1-sulfonamide (ML-60218; Calbiochem). The columns were washed twice with 12 mL of buffer WA (10 mM Tris, pH 6.3, 15% ethanol, 300 mM KCl, and 0.15% Triton X-100). The RNA was precipitated with 10 mL of buffer EL (10 mM Tris, pH 6.3, 15% ethanol, 650 mM KCl) into 2.5 vol. of 99.6% ethanol. The tRNA was precipitated o/n at −20°C and pelleted by centrifugation for 20 min at 10,000g. Residual salt was removed by washing the pellet three times with 80% ethanol. The tRNA pellet was dried at room temperature for ~10 min and resuspended into 50 µL of RNase-free water.

Inhibition of tRNA synthesis

To assess turnover of nucleoside modifications, tRNA synthesis was inhibited by the addition of RNA polymerase inhibitors. S288C cultures at 37°C were grown to OD600 = 0.6 as described above, at which point the cultures were supplemented with either 5 µg/mL thiolutin (Sigma-Aldrich) or 50 µg/mL N1-[3-(5-chloro-3-methylbenzo[b]thiophen-2-yl)-1-methyl-1H-pyrazol-5-yl]-2-chlorobenzene-1-sulfonamide (ML-60218; Calbiochem). The rpo31-698 (Li et al. 2011) culture was grown to OD600 = 0.6 at 30°C and subsequently shifted to 37°C. The cultures were grown for an additional 6 h and samples for total RNA isolation were collected at indicated time points. Total RNA isolation was performed as described above and precipitated by adding 2.5 vol. of 99.6% ethanol to the final aqueous phase. The total RNA was precipitated o/n at −20°C and washed with 80% ethanol.

Enzymatic digestion of tRNA and nucleoside purification

D Dephosphorylated mononucleosides were generated by enzymatic digestion of 25 µg tRNA using 80 milliunits of Nuclease P1 from Penicillium citrinum (Sigma-Aldrich) in the presence of 0.2 unit of Shrimp Alkaline Phosphatase (Fermentas) in 30 µL reactions at 37°C containing 2 mg/mL ZnCl2 and 1× NEB3 buffer. Following 1.5 h incubation, the reaction mixture was supplemented with 15 µL 0.5 M ammonium bicarbonate and incubation was resumed at 37°C for 1 h. The reaction was terminated by adding 5.0%
trifluoroacetic acid (TFA) in water to a final concentration of 1.0%. Nucleosides were purified with HyperSep Hypercarb Spin Tips (Thermo Scientific). Briefly, the spin tips were equilibrated three times with 50 μL 0.1% TFA in H2O. The digested samples were applied and washed three times with 50 μL 0.1% TFA in H2O. The nucleosides were eluted twice with 50 μL 0.1% TFA in 80% acetonitrile. All centrifugation steps were carried out in a microcentrifuge for 30 sec to 1 min at 845g. The samples were dried to completion in a Savant SpeedVac concentrator (Thermo Scientific) and resuspended in either 0.1% formic acid (for chromatography analysis) or 5 mM sodium acetate (for mass spectrometry).

**Reverse-phase high-performance liquid chromatography of nucleosides**

Analysis of nucleosides was performed on a Hypercarb 3 μm 2.1 × 150 mm porous graphitic carbon column (Thermo Scientific) using a Knauer PLATINblue UPLC system with a PDA-1 photodiode array detector (Dr. Ing. Herbert Knauer GmbH). The column was equilibrated in 0.1% formic acid with 2% acetonitrile at a flow rate of 0.75 mL/min until a stable baseline was achieved. Twenty-five micrograms of nucleoside digest was loaded onto the column and washed for 5 column volumes (CV) with 2% acetonitrile. Separation was obtained by applying a linear gradient from 10% to 40% acetonitrile at 0.75 mL/min for 40 CV, followed by a wash at 98% acetonitrile and regeneration at 2% acetonitrile for 5 CV, respectively. Absorption at 260 nm was recorded.

The average retention times for all nucleotide modifications were obtained by analyzing synthetic nucleoside standards (Carbosynth Ltd.), mcm5U, mcm5s2U, ncm5U, and ncm5s2U were a generous gift by Andrzej Malkiewicz. Nucleoside modification levels were determined using automated peak analysis in ChromGate 3.3.2 (Dr. Ing. Herbert Knauer GmbH) by normalization of the signal intensity to adenosine in each run.

**Liquid chromatography mass spectrometry**

Nucleosides were resuspended in 25 μL of 5 mM sodium acetate buffer pH 5.3 and subjected to LC–MS analysis using a Proxenon EASY nLC online coupled via a C18 reversed-phase column (Synergi 4u Hydro-RP 80A 0.3 × 150 mm, Phenomenex Ltd.) and a stainless steel emitter (Proxenon, 0.03 × 40 mm) to a Q Exactive mass spectrometer (Thermo Finnigan). The column was attached to a nano ESI source from Proxenon. Injection volume was 5 μL. The buffer system consisted of 5 mM sodium acetate pH 5.3 (Buffer A) and 40% acetonitrile (Buffer B). Nucleosides were separated using a multistep gradient (0%–1% B in 5 min; 1%–2% B in 4:20 min; 2%–3% B in 1:40 min; 3%–5% B in 2 min; 5%–25% B in 19 min; 25%–50% in 6 min; 50%–75% B in 5:30 min; hold at 75% for 1:30 min; 75%–99% B in 5 min; hold at 99% for 7 min) at a flow rate of 1 μL/min. The mass spectrometer was run in the positive mode at a resolution of 70,000. Full MS spectra were recorded in profile mode in the scan range from m/z 100–700, with the AGC target value set to 1 × 105 and the maximum fill time to 200 msec. Only single charged ions were allowed ([Nuc+H]+), excluding uncharged compounds and compounds with z ≥ 2. In source, CID fragmentation was enabled (5.0 eV) to allow for incomplete fragmentation of most nucleosides at the glycosidic bond between the ribose and the corresponding base. Loss of the sugar is reflected by a mass difference of 132.04 (ribosyl) or 146.06 (methylated ribosyl) between the intact nucleoside and remaining base, for both of which signals are present in the MS spectrum. This mass loss is characteristic for ribonucleosides and simplified identification of the nucleoside spectra in complex mixtures. Average retention times were obtained as described above.

**Automated quantification of nucleoside modifications**

Identification and quantification of nucleoside modifications was performed using pyQms (Barth et al. 2014). In brief, pyQms generates high accuracy isotopologues from chemical formulas and matches those onto MS1 spectra using a weighted similar match score (Gower 1971). A manually curated list of all potentially expected nucleosides was used as input for pyQms (data obtained from MODOMICS) (Mechnicka et al. 2013). The isotopologues were grouped into five classes that share the same nitrogenous base and differ only by one of the following neutral loss mass: ribose (C5O4H8), methylated ribose (C6O4H10), ribophosphate (C6O7H11P1), methylated ribophosphate (C6O8H12P1), or phosphate (H2O3P1). When insource fragmentation is applied, multiple members of one class can be detected in the same MS1 scan. This allows us to distinguish molecules that share the same chemical formula, yet differ in their chemical compositions after neutral loss.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available for this article.

**ACKNOWLEDGMENTS**

We gratefully thank Prof. Andrzej Malkiewicz for synthesis of modified nucleosides, and Dr. Tamara Bar-Magen Numhauser for skilled assistance in MS data analysis. This work was supported by grants from the Max Planck Society (Max-Planck-Gesellschaft), the North Rhine-Westphalian Ministry for Innovation, Science and Research [314-400 010 09], and the European Research Council [ERC-2012-StG 310489-tRNAmodi] (to S.A.L.). L.P.S. is a Sigrid Jusélius Fellow (2012–2014). F.A. is supported by the International Max Planck Research School—Molecular Biomedicine.

Received September 20, 2014; accepted November 18, 2014.

**REFERENCES**

Agriq PF, Koh H, Söll D. 1973. The effect of growth temperatures on the in vivo ribose methylation of Bacillus stearothermophilus transfer RNA. *Arch Biochem Biophys* 154: 277–282.

Barth J, Bergner SV, Jaeger D, Niehues A, Schulze S, Scholz M, Fufezan C. 2014. The interplay of light and oxygen in the reactive oxygen stress response of Chlamydomonas reinhardtii dissected by quantitative mass spectrometry. *Mol Cell Proteomics* 13: 969–989.

Bauer F, Matsuyama A, Candiracci J, Dieu M, Scheliga J, Wolf DA, Yoshida M, Hermand D. 2012. Translational control of cell division by Elongator. *Cell Rep* 1: 424–433.

Begley U, Dyavaiah M, Patil A, Rooney JP, DiRenzo D, Young CM, Conklin DS, Zitomer RS, Begley TJ. 2007. Trm9-catalyzed tRNA modifications link translation to the DNA damage response. *Mol Cell* 28: 860–870.
Björk GR, Jacobsson K, Nilsson J, Johansson MJ, Byström AS, Persson OP. 2001. A primordial tRNA modification required for the evolution of life? *EMBO J* 20: 231–239.

Björk GR, Huang B, Persson OP, Byström AS. 2007. A conserved modified wobble nucleoside (mcm-S’U) in lysi-tRNA is required for viability in yeast. *RNA* 13: 1245–1255.

Chan CT, Dyavaiah M, DeMott MS, Taghizadeh K, Dedon PC, Bogley TJ. 2010. A quantitative systems approach reveals dynamic control of tRNA modifications during cellular stress. *PLoS Genet* 6: e1001247.

Chan CT, Pang YL, Deng W, Babu IR, Dyavaiah M, Byström TJ, Dedon PC. 2012. Reprogramming of tRNA modifications controls the oxidative stress response by codon-biased translation of proteins. *Nat Commun* 3: 937.

Crück FH, Griffith JS, Orgel LE. 1957. Codes without commas. *Proc Natl Acad Sci* 43: 416–421.

Dudley E, Tytten R, Bond A, Lemiére F, Brenton AG, Esmans EL, Newton RP. 2005. Study of the mass spectrometric fragmentation of pseudouridine: comparison of fragmentation data obtained by matrix-assisted laser desorption/ionisation post-source decay, electrospray ion trap mass spectrometry, and by a method utilising electrospray quadrupole time-of-flight tandem mass spectrometry and in-source fragmentation. *Rapid Commun Mass Spectrom* 19: 3075–3085.

Emilsson V, Kurland CG. 1990. Growth rate dependence of transfer RNA abundance in *Escherichia coli*. *EMBO J* 9: 4359–4366.

Esberg A, Huang B, Johansson MJ, Byström AS. 2006. Elevated levels of two tRNA species bypass the requirement for elongator complex in transcription and exocytosis. *Mol Cell* 24: 139–148.

Fernández-Vázquez J, Vargas-Pérez I, Sansó M, Buhne K, Carmona M, Paulo E, Hermad D, Rodríguez-Gallego M, Ayte J, Leidel S, et al. 2013. Modification of tRNA<sup>35</sup>UUC by elongator is essential for efficient translation of stress mRNAs. *PLoS Genet* 9: e1003647.

Gabriel AP, Spellman PT, Kao CM, Carmel-Harel O, Eisen MB, Storz G, Helm M, Alfonzo JD. 2014. Posttranscriptional RNA modifications: two tRNA species bypass the requirement for elongator complex in translation and exocytosis. *RNA* 20: 1741–1752.

Grosjean H, Breton M, Sirand-Pugnet P, Tardy F, Thiaucourt F, Citti C, Jalluz R, Policarpo M, Le Breton J, Kieffer J, et al. 2013. Influence of temperature on tRNA modification genes required for cell growth at extremely high temperatures. *Mol Biol Cell* 24: 18255–18260.

Igloi GL. 1988. Interaction of tRNAs and of phosphorothioate-substituted nucleotides with an organomercurial. Probing the chemical environment of thiolated residues by affinity electrophoresis. *Biochemistry* 27: 3842–3849.

Iwasaki O, Tanaka Y, Tanizawa H, Grewal SI, Noma K. 2010. Thermally induced biosynthesis of 2′-O-methylguanosine in tRNA from an extreme thermophile, *Thermus thermophilus* HB827. *Proc Natl Acad Sci* 77: 1922–1926.

Laxman S, Sutter BM, Wu X, Kumar S, Guo X, Trudgian DC, Mirzaei H, Tu BP. 2013. Sulfur amino acids regulate transcriptional capacity and metabolic homeostasis through modulation of tRNA thiolation. *Cell* 154: 416–429.

Leidel S, Pedrioli PG, Bucher T, Brost R, Costanzo M, Schmidt A, Aebersold R, Boone C, Hofmann K, Peter M. 2009. Ubiquitin-related mediator Umri acts as a sulphur carrier in thiolation of eukaryotic transfer RNA. *Nature* 458: 228–232.

Li Z, Vizeacoumar FJ, Bahr S, Li J, Warringer J, Vizeacoumar FS, Min R, Vandersluis B, Bellay J, Devit M, et al. 2011. Systematic exploration of essential yeast gene function with temperature-sensitive mutants. *Nat Biotechnol* 29: 361–367.

Liti G, Carter DM, Moses AM, Warringer J, Part L, James SA, Davey RP, Roberts IN, Burt A, Koufopanou V, et al. 2009. Population genomics of domestic and wild yeasts. *Nature* 458: 337–341.

Machnicka MA, Milanowska K, Osman Ogolu O, Purta E, Kurkowska M, Ochowiak A, Januszewski W, Kalinowski S, Dunin-Horkawicz S, Rother KM, et al. 2013. MODOMICS: a database of RNA modification pathways—2013 update. *Nucleic Acids Res* 41: D262–D267.

McCusker JH, Clemons KV, Stevens DA, Davis RW. 1994. *Sarcosarcymo- cyes cerevisiae* virulence phenotype as determined with CD-1 mice is associated with the ability to grow at 42°C and form pseudohyphae. * Infect Immun* 62: 5447–5455.

Nakai Y, Nakai M, Hayashi H. 2008. Thio-modification of yeast cytosolic tRNA requires a ubiquitin-related system that resembles bacterial sulfur transfer systems. *J Biol Chem* 283: 27469–27476.

Noma A, Sakaguchi Y, Suzuki T. 2009. Mechanistic characterization of the sulfur-relay system for eukaryotic 2-thiouridine biogenesis at tRNA wobble positions. *Nucleic Acids Res* 37: 1335–1352.

Nooon KR, Guyron R, Crain PF, McCloskey JA, Thomm M, Lim J, Cavacchioli R. 2003. Influence of temperature on tRNA modification in archaea: Methanococccoides burtonii (optimum growth temperature [T<sub>opt</sub>], 23°C) and *Settoria hydrogenophila* (T<sub>opt</sub>, 95°C). *J Bacteriol* 185: 5483–5490.

Phizicky EM, Alfonzo JD. 2010. Do all modifications benefit all tRNAs? *FEBS Lett* 584: 265–271.

Phizicky EM, Hopper AK. 2010. tRNA biology charges to the front. *Genes Dev* 24: 1832–1860.

Rhind N, Chen Z, Yassour M, Thompson DA, Haas BJ, Habib N, Wapinski I, Roy S, Lin MF, Heiman DI, et al. 2011. Comparative functional genomics of the fission yeasts. *Science* 332: 930–936.

Rodriguez-Hernandez A, Spears JL, Gaston KW, Limbach PA, Gamper H, Hou YM, Kaiser R, Agris PF, Perona J, et al. 2013. Structural and mechanistic basis for enhanced translational efficiency by 2-thiouridine modification in tRNA. *J Mol Biol* 425: 3888–3906.

Schleier CD, Van der Veen AG, Damon JR, Spooner E, Ploegh HL. 2008. A functional proteomics approach links the ubiquitin-related modifier Umr1 to a tRNA modification pathway. *Proc Natl Acad Sci* 105: 18255–18260.

Shigi N, Sakaguchi Y, Suzuki T, Watanabe K. 2006. Identification of two tRNA thiolation genes required for cell growth at extremely high temperatures. *J Biol Chem* 281: 14306–14308.

Tipper DJ. 1973. Inhibition of yeast ribonucleic acid polymerases by thiolutin. *J Biol Chem* 248: 245–256.

Vivancos AP, Jara M, Zuin A, Sanso M, Hidalgo E. 2006. Oxidative stress in *Schizosaccharomyces pombe*: different H<sub>2</sub>O<sub>2</sub> levels, different responses. *PLoS Genet* 2: 3085–3087.

Vivancos AP, Jara M, Zuin A, Sanso M, Hidalgo E. 2006. Oxidative stress in *Schizosaccharomyces pombe*: different H<sub>2</sub>O<sub>2</sub> levels, different responses. *PLoS Genet* 2: 3085–3087.

Watanabe K, Shinma M, Oshima T, Nishimura S. 1976. Heat-induced thiolation of yeast cytosol. *Bacteriol Rev* 40: 337–341.

Watanabe K, Yoshida K, Nakamura M, Hamano M, Endo H. 2006. Oxidative stress in *Schizosaccharomyces pombe*: different H<sub>2</sub>O<sub>2</sub> levels, different responses. *PLoS Genet* 2: 3085–3087.

Watanabe K, Shimna M, Oshima T, Nishimura S. 1976. Heat-induced stability of tRNA from an extreme thermophile, *Thermus thermophilus*. *Biochem Biophys Res Commun* 72: 1137–1144.

Wu L, Pan J, Thoroddsen W, Wysong DR, Blackman RK, Boluwa CE, Gould AE, Ocin TD, Dick LR, Errada P, et al. 2003. Novel small-molecule inhibitors of RNA polymerase III. *Eukaryot Cell* 2: 256–264.

Zinshteyn B, Gilbert WV. 2013. Loss of a conserved tRNA anticodon modification perturbs cellular signaling. *PLoS Genet* 9: e1003675.