Disruption of MRG19 results in altered nitrogen metabolic status and defective pseudohyphal development in *Saccharomyces cerevisiae*

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It was previously shown that MRG19 downregulates carbon metabolism in *Saccharomyces cerevisiae* upon glucose exhaustion, and that the gene is glucose repressed. Here, it is shown that glucose repression of MRG19 is overcome upon nitrogen withdrawal, suggesting that MRG19 is a regulator of carbon and nitrogen metabolism. β-Galactosidase activity fostered by the promoter of GDH1/3, which encode anabolic enzymes of nitrogen metabolism, was altered in an MRG19 disruptant. As compared to the wild-type strain, the MRG19 disruptant showed a decrease in the ratio of 2-oxoglutarate to glutamate under nitrogen-limited conditions. MRG19 disruptants showed reduced pseudohyphal formation and enhanced sporulation, a phenomenon that occurs under conditions of both nitrogen and carbon withdrawal. These studies revealed that MRG19 regulates carbon and nitrogen metabolism, as well as morphogenetic changes, suggesting that MRG19 is a component of the link between the metabolic status of the cell and the corresponding developmental pathway.

INTRODUCTION

Micro-organisms are endowed with the genetic capability to respond to frequently changing carbon and/or nitrogen deprivation by undergoing unique developmental changes. In *Saccharomyces cerevisiae*, deprivation of carbon and/or nitrogen elicits changes in metabolism and development to circumvent the nutritional deprivation (Esposito & Klapholz, 1981; Werner-Washburne et al., 1996; Gancedo, 2001). Diploid cells of *S. cerevisiae* undergo sporulation when carbon and nitrogen supply is limited (Esposito & Klapholz, 1981), whereas they put forth pseudohyphae in the absence of nitrogen but in the presence of glucose (Lorenz et al., 2000). These developmental changes, which are due to a combination of signals occurring in response to carbon and nitrogen withdrawal, indicate that the regulation of carbon and nitrogen metabolism is in some way interconnected. GLN3 responds to the low-nitrogen signal, and activates the transcription of nitrogen catabolite-repressed genes (Cunningham et al., 1996); mutation in this gene leads to an inability to put forth pseudohyphae (Lorenz & Heitman, 1998a, b). Recently, it was shown that GLN3 is also regulated by the glucose-responsive SNF1-kinase-mediated phosphorylation mechanism (Bertram et al., 2002; Cox et al., 2002); thus, it is evident that the regulation of carbon and nitrogen metabolism is interdependent. While it is clear that a unique collection of metabolic signals are required to induce specific morphogenetic changes, the underlying metabolic basis that predisposes the cell to opt for one or the other developmental programme is not clear.

MRG19 was isolated as a multi-copy repressor of galactose toxicity (Kabir et al., 2000). It was observed that disruption of MRG19 resulted in the derepression of CYC1 under conditions of carbon limitation (Khanday et al., 2002). Based on this, it was suggested that in a wild-type strain, the function of MRG19 is to regulate the oxidation of limited carbon so as to channel it for biosynthesis. The protein is localized in the nucleus and its production is repressed in the presence of glucose in an MIG1-dependent fashion (Khanday et al., 2002). Genome-wide expression analysis revealed that MRG19 transcript levels increase upon nitrogen starvation (Gasch et al., 2000), even in the presence of glucose. MRG19 is the only gene that escapes MIG1-dependent glucose repression upon nitrogen starvation, suggesting that MRG19 plays a role in the link between carbon and nitrogen metabolism. In this study, we demonstrate that MRG19 regulates the expression of NADP-glutamate dehydrogenases, which are the key enzymes of ammonia assimilation, encoded by GDH1/3. Upon nitrogen deprivation, the ratio of steady-state levels of 2-oxoglutarate to glutamate increased in the wild-type strain as compared to the MRG19 disruptant. The MRG19 disruptant was defective in the formation of pseudohyphae. The implications of these results are discussed in the context of the metabolic basis of development.
METHODS

Media and growth conditions. Yeast strains were grown at 30 °C in YEPD or synthetic dropout or complete media as described. Either 2 % (w/v) glucose, or 3 % (v/v) potassium lactate pH 5.7 with 2 % (v/v) glycerol was used, as indicated. Yeast transformation was carried out as described by Ito et al. (1983). Low-ammonia medium (SLAD) was prepared as described by Gimeno & Fink (1992) and Gimeno & Fink (1994), and it was used for scoring pseudohyphal growth. Amino acids were added as necessary at a concentration of 200 mg l⁻¹ as indicated. Strains scored for pseudohyphal filament formation were streaked onto SLAD agar, and observed after 72 h of growth at 30 °C. The sporulation medium was used as described by Sherman (1991).

Strains. DNA manipulation was carried out in Escherichia coli strain XL-1 Blue, as described by Sambrook et al. (1989). Yeast strains are listed in Table 1. The MRG19-disrupted yeast strain YO5449 was obtained from Euroscarf (Institute of Microbiology, Johann Wolfgang Goethe University, Frankfurt, Germany) and its isogenic wild-type strain YO5449WT was obtained by crossing YO5449 with Y10345, followed by screening for genetic-in-scoring haploids. The wild-type and its isogenic MRG19-disrupted strains were crossed with isogenic ScPJB644-L (wild-type) and ScPJB644-19A (MRG19-disrupted) to obtain YO5449WT/ScPJB644-L and YO5449/ScPJB644-19A, respectively.

Plasmids and plasmid constructions. The GDH1–lacZ fusion plasmid p426, which is a ura3-based episomal plasmid, was obtained from Dr B. Daignan-Fornier (Daignan et al., 1996). The construct pGDS2 was derived from p77-2 (Miller & Magasanik, 1991). A 1 kb fragment of GDH3 5′ untranslated region was amplified using the primers PJB119 5′-GCC CGG AGA TCT TAA AAA CGG TCA AGG CAT TTA CGC-3′ and PJB120 5′-GCC CGG GTG GAC TAC TTA CTT ACC TTT TCT TTT-3′ and cloned as a BglI–SalI insert into pGDS2 digested with BglII and SalI. Plasmid pKS19 is a multi-copy plasmid bearing MRG19 under its own promoter, as described previously (Kabir et al., 2000). Sad–SalI and Sal–SalI fragments isolated from pKDS19 were cloned into SacI–SalI-digested YCPyac33 to get single-copy MRG19 plasmid YCPyac19 (Khanday et al., 2002). YCPyac19–PstI, used as a vector control, was derived from YCPyac19 by digesting it with PstI to remove most of the ORF of MRG19 and religated.

Quantification of pseudohyphal cells. Pseudohyphal cells were quantified as described by Mosch et al. (1996). Strains were grown on SLAD medium at 30 °C for 3 days. The noninvasive cells were removed by washing the agar plates with water, and the invasive cells were scraped off the agar. The cells were then suspended in 50 ml water, and analysed for cell shape by light microscopy. The length to width ratio of the cells was determined, and the cells were classified into two groups: yeast-form cells with a length to width ratio less than two, and pseudohyphal cells with a length to width ratio greater than two. A minimum number of 50 cells was analysed in each experiment, and the percentage of pseudohyphal cells in the population studied was determined. The results presented are means of three individual experiments, and the level of significance (t = 29.029, d.f. = 2 and P value = 0.0012) was estimated by calculating paired t-test and P values as described by Freund & Wilson (1993).

Image analysis. A Leica DM LM microscope was used for all microscopic analyses. The images were analysed using Image Analysis Software from Soft Imaging Systems.

Western blot analyses. Cells were harvested and extracts were prepared as described previously (Khanday et al., 2002). Western blot analyses were carried out as described previously (Khanday et al., 2002). Protein was estimated as described by Bradford (1976). All the experiments were repeated at least three times. As a control, Western blots of the cell extracts were also carried out using glucose-6-phosphate dehydrogenase (Zwf1p) antiserum (Sigma).

β-Galactosidase assays. β-Galactosidase activity was assayed in cell extracts, as described by Adams et al. (1997). Duplicate samples were taken for each determination. Experiments were repeated at least three times with a different transformant each time. Specific activities are represented as nmol product min⁻¹ (mg protein)⁻¹.

Table 1. Yeast strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference/source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y10345</td>
<td>MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 YAL062w::kanMX4</td>
<td>Euroscarf</td>
</tr>
<tr>
<td>Y05449</td>
<td>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YPR030w::kanMX4</td>
<td>Euroscarf</td>
</tr>
<tr>
<td>Y05449WT</td>
<td>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</td>
<td>This study</td>
</tr>
<tr>
<td>ScPJB644-L</td>
<td>MATa ade2 ura3-32 trp1 LEU2</td>
<td>Khanday et al. (2002)</td>
</tr>
<tr>
<td>ScPJB644-19A</td>
<td>MATa ade2 ura3-32 leu2-3, 112 trp1 mrg19::LEU2</td>
<td>Khanday et al. (2002)</td>
</tr>
<tr>
<td>Y05449WT/ScPJB644-L</td>
<td>MATa/MATa ADE2/ade2 his3Δ1/HIS3 ile/ILE leu2Δ0/LEU2 met15Δ0Met15 trp1/1TRP1 ura3Δ0/ura3-32</td>
<td>This study</td>
</tr>
<tr>
<td>YO5449/ScPJB644-19A</td>
<td>MATa/MATa ADE2/ade2 his3Δ1/HIS3 ile/ILE leu2Δ0/LEU2 met15Δ0Met15 trp1/1TRP1 ura3Δ0/ura3-32</td>
<td>This study</td>
</tr>
<tr>
<td>MS218</td>
<td>MATa ura3-32</td>
<td>Stanbrough et al. (1995)</td>
</tr>
<tr>
<td>PM71</td>
<td>MATa ura3-32 leu2-3,112 glu3Δ5::LEU2</td>
<td>Stanbrough et al. (1995)</td>
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</table>
**Results**

MrG19p levels increase upon nitrogen depletion

*MRG19* transcript levels increase under conditions of nitrogen depletion (Gasch *et al.*, 2000). To determine whether an increase in transcript levels reflects an increase in protein levels, Western blot analysis was carried out on extracts obtained from cells grown in SLAD (nitrogen-depleted with glucose as carbon source) medium. MrG19p was expressed in SLAD medium, but not in synthetic complete medium with glucose as the carbon source (Fig. 1a, compare lanes 2 and 3). In light of the observation that *MRG19* is glucose repressed (Khanday *et al.*, 2002; and results presented in Fig. 1a, lane 2), our results indicate that *MRG19* expression in the nitrogen-depleted condition overrides glucose repression. Based on this, we suggest that *MRG19* is a key player under conditions of carbon or nitrogen starvation. MEP2, encoding a high-affinity ammonia permease, is the only known gene whose transcription is activated upon nitrogen starvation in a GLN3-dependent fashion (Bertram *et al.*, 2002; Cox *et al.*, 2002). We found the presence of putative Gln3p binding sites in the promoter region of *MRG19*; however, we observed that expression of *MRG19* is independent of Gln3p in nitrogen-depleted media (Fig. 1b).

Expression of GDH1 and GDH3 is dependent on MRG19

Since the synthesis of glutamate from 2-oxoglutarate and ammonia, catalysed by NADP-glutamate dehydrogenase, links carbon and nitrogen metabolism, we wanted to study the effect of *MRG19* on this pathway. GDH1 and GDH3 encode major and minor isozymes of NADP-glutamate dehydrogenase, respectively (Moye *et al.*, 1985; Avendano *et al.*, 1997). To assess the effect of MrG19p on the expression of these enzymes, we first compared GDH1 and GDH3 promoter activities in the *MRG19* disruptant versus the wild-type strain when cells were grown in glycerol plus lactate as the carbon source under nitrogen-sufficient conditions. GDH1 and GDH3 promoter-driven β-galactosidase activity decreased to 33% and 47%, respectively, in the *MRG19*-disrupted strain as compared to the wild-type (Fig. 2a, b; lanes 1 and 2) when glycerol plus lactate was used as the carbon source. When cells were grown with glucose as carbon source in the presence of sufficient nitrogen, no significant difference was observed in GDH1 activity.
and GDH3 promoter activity in the exponential phase. This result is in agreement with the observation that MRG19 is not expressed in the presence of glucose under sufficient nitrogen (Fig. 2a, b, lanes 3 and 4). However, in late exponential phase, GDH1 showed an increase in promoter activity in the wild-type as compared to the MRG19 disruptant (Fig. 2a, lanes 5 and 6). It is known that MRG19 expression begins to appear as the cells enter the diauxic phase (Gasch et al., 2000), and this could be the reason for the observed difference. Since GDH3 expression is negligible in glucose-grown cells, we were unable to observe the difference even if it existed (Fig. 2b, lanes 5 and 6). Having ascertained that GDH1/3-promoter-driven β-galactosidase activities reflect the involvement of Mrg19p in the expression of GDH1/3, we wanted to see the effect of MRG19 disruption on GDH1/3 under the nitrogen-deprivation condition. GDH1, but not GDH3, promoter-driven β-galactosidase activity was reduced to 56 % (Fig. 2a, b; lanes 7 and 8) in an MRG19-disrupted strain. The results suggest that MRG19 activates the expression of GDH1 in response to nitrogen depletion in the presence of glucose as the carbon source.

Glutamate/2-oxoglutarate levels are altered in the MRG19-disrupted strain under nitrogen-deprivation conditions

To assess the functional significance of the differential expression of GDH1 in an MRG19-disrupted background under nitrogen deprivation, we monitored the steady-state levels of glutamate and 2-oxoglutarate. The steady-state levels of 2-oxoglutarate and glutamate in the MRG19-disrupted strain increased as compared to the wild-type when grown in glycerol/lactate under nitrogen-sufficient conditions (Fig. 3a, b; lanes 1 and 2). However, in the MRG19-disrupted strain, the steady-state level of 2-oxoglutarate decreased to about 62 % of that of the wild-type, while the steady-state levels of glutamate remained the same in SLAD medium (Fig. 3a, b; lanes 3 and 4).

The MRG19-disrupted strain shows reduced pseudohyphal formation upon nitrogen starvation

The above results suggest that MRG19 is a component of the cellular machinery which responds to nitrogen starvation.
and alters the expression of \textit{GDH1} and \textit{GDH3}, which encode the key enzymes in ammonia assimilation. In \textit{Benjaminiella poitrasii}, dimorphic transition is correlated with differential expression of different NADP-glutamate dehydrogenases and the carbon/nitrogen balance (Khale \textit{et al}., 1992). Based on this information, we studied the role of \textit{MRG19} in the formation of pseudohyphae. The \textit{MRG19}-disrupted homo-diploid strain transformed with vector control (YCP19–\textit{PstI}) showed reduced pseudohyphal formation as compared to the corresponding transformants of the wild-type (Fig. 4a). Upon transformation of the wild-type with YCP19 (single-copy \textit{MRG19}), we observed that pseudohyphal formation increased over and above that observed on transformation with the corresponding vector control. As expected, pseudohyphal formation was reduced significantly when the concentration of ammonium sulphate was increased tenfold (Fig. 4b), but the difference in pseudohyphae between the wild-type and the \textit{MRG19} disruptant was still discernible. The difference in the pseudohyphal formation between the wild-type and the \textit{MRG19} disruptant was determined as described in Methods. The \textit{MRG19} disruptant showed a 44% decrease in the number of pseudohypal cells as compared to the wild-type. Based on these results, we infer that the \textit{MRG19} disruptant is impaired in pseudohyphal formation. 

\textbf{MRG19 disruption increases sporulation efficiency}

Genome-wide expression analysis showed that \textit{MRG19} transcript levels decrease during sporulation (Chu \textit{et al}., 1998). This suggests that some relationship exists between

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig4.png}
\caption{Pseudohyphal formation on nitrogen starvation in the wild-type strain and the \textit{MRG19}-disrupted diploid strain. Pseudohyphal development was determined in SLAD medium containing 0.05 mM (a) and 0.5 mM (b) ammonium sulphate in the wild-type (YO5449WT/ScPJB644) and the \textit{MRG19}-disrupted (YO5449/ScPJB644-19\textit{Δ}) strains transformed with the vector control (Ycp19–\textit{PstI}) or single-copy \textit{MRG19} (YCP19). Two independent colonies from each group are shown.}
\end{figure}
MRG19 and sporulation. In order to analyse this, we monitored sporulation efficiency in isogenic wild-type and MRG19-disrupted diploid strains. The percentage sporulation of both strains was calculated as described in Methods. The percentage sporulation in the wild-type strain was 12%, while that in the MRG19-disrupted strain was 38%. It is clear from the data that disruption of MRG19 resulted in a 68% increase in sporulation efficiency over the wild-type.

DISCUSSION

The key reaction in the assimilation of nitrogen to glutamate is catalysed by NADP-glutamate dehydrogenase under conditions of excess ammonia. Under conditions of limited ammonia, or in the absence of NADP-glutamate dehydrogenase, the glutamine synthetase (GS)–glutamate synthase (GOGAT) pathway functions to produce glutamate (Avendano et al., 1997). S. cerevisiae is unique in that it has two isozymes of NADP-glutamate dehydrogenase, GDH1 and GDH3, which are expressed in glucose and ethanol, respectively (Moye et al., 1985; Avendano et al., 1997). Regulation of the expression of GDH1 is regulated by a complex interplay of many transcriptional regulators (Dang et al., 1996; Riego et al., 2002). Here, we demonstrate that in the presence of sufficient ammonium sulphate, both GDH1 and GDH3 are positively regulated by MRG19 when cells are grown in a non-fermentable carbon source, but not in the presence of glucose. Absence of MRG19-dependent regulation of GDH1/3 in glucose-grown cells is consistent with the observation that MRG19 is not expressed in the presence of glucose. This makes physiological sense, in that when cells are growing in glucose, the major fate of 2-oxoglutarate is to enter the metabolic pool, since it cannot be oxidized. This is unlike the situation when cells are grown in metabolically inferior carbon sources, such as glycerol and ethanol, when 2-oxoglutarate has to enter oxidation and the biosynthetic pool. Activation of GDH1/3 by MRG19 probably optimizes the carbon utilization for biosynthetic and oxidative purposes. This view is supported by our earlier observation that MRG19 represses CYC1 activity (Khanday et al., 2002). While the mechanism of regulation of GDH1 has been studied in significant detail, our knowledge regarding the mechanism of regulation of expression of GDH3 is negligible. In this study, we demonstrate that expression of GDH3 is regulated in an MRG19-dependent fashion.

The ratio of 2-oxoglutarate to glutamate in the wild-type (1.85) and the MRG19 disruption strain (1.35) grown in glycerol/lactate with sufficient ammonium sulphate did not change significantly (although the absolute amounts increased approximately twofold). The levels of 2-oxoglutarate reported here are comparable with those reported elsewhere (DeLuna et al., 2001). In SLAD medium, the ratio of 2-oxoglutarate to glutamate in the wild-type (8.13) is higher than that in the MRG19-disrupted strain (4.4). This is mainly due to a decrease in 2-oxoglutarate [from 30 to 18.6 nmol (mg protein)−1] rather than to an increase in the glutamate level [3.69 to 4.2 nmol (mg protein)−1] in the MRG19 disruptant, as compared to the wild-type. Therefore, the only discernible difference is in the level of 2-oxoglutarate in the MRG19 disruptant as compared to the wild-type strain in SLAD medium. This increase, however, does not agree with the observation that GDH1/3 levels showed a decrease in the MRG19-disrupted strain under this condition. It is possible that under nitrogen-insufficient conditions, the GS–GOGAT pathway could be more active in MRG19 deletion, thereby reducing the level of 2-oxoglutarate. In addition to this, the retrograde (RTG) system is responsible for providing 2-oxoglutarate for glutamate synthesis in the presence of glucose at low glutamate levels (Parikh et al., 1987; Liao et al., 1991; Liu & Butow, 1999). It is possible that in an MRG19-deletion strain, the RTG system may not be as active as in a wild-type strain. These observations suggest that in a wild-type strain, the ratio of 2-oxoglutarate to glutamate in the nitrogen-depleted condition is significantly higher than that in the nitrogen-sufficient condition. It is clear from our data that this ratio decreases in an MRG19-disrupted strain as compared to the wild-type upon nitrogen depletion. From this, it appears that the MRG19-disrupted strain tends to mimic the nitrogen-sufficient condition upon nitrogen depletion. This difference between the strains in the nitrogen-depleted condition correlates with the decreased ability of the MRG19-disrupted strain to put forth pseudohyphae. Studies have shown that Mrg19p forms a complex with Bmh1/2p (Ho et al., 2002), which is known to be essential for pseudohyphal formation (Roberts et al., 1997), suggesting that it could also modulate the signal transduction pathway for pseudohyphal formation.

It is interesting to note that MRG19 expression is switched off when the cell encounters sufficient carbon and nitrogen, and is activated upon carbon or nitrogen starvation. However, when both carbon and nitrogen are depleted, expression of MRG19 is repressed. In the light of the observation that MRG19 is repressed upon sporulation, and our results that disruption of MRG19 increases sporulation, we suggest that MRG19 acts as a suppressor of sporulation. However, the effects of MRG19 disruption on different parameters are moderate, suggesting that redundant pathways regulate the response to limited nutrients. This makes it difficult to assign a specific role to MRG19. Our studies do not provide a mechanistic insight into whether the metabolic perturbation and phenotypic changes observed with respect to MRG19 have a cause and effect relationship, but unravel a hitherto unknown link between the metabolic status of the cell and the corresponding developmental pathway. These observations provide an avenue to further explore the connection between carbon and nitrogen metabolism.

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Saccharomyces cerevisiae previously unrecognized route for glutamate biosynthesis in assimilation in J Bacteriol pathway regulation between nitrogen and carbon metabolisms.


REFERENCES


