Phenotypic consequences of purine nucleotide imbalance in

Saccharomyces cerevisiae

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ABSTRACT

Coordinating homeostasis of multiple metabolites is a major task for living organisms and complex interconversion pathways contribute to achieve proper balance of metabolites. AMP deaminase (AMPD) is such an interconversion enzyme that allows IMP synthesis from AMP. In this paper, we show that, under specific conditions, lack of AMPD activity impairs growth. Under these conditions, we found that the intracellular guanylic nucleotide pool was severely affected. *In vivo* studies of two AMPD homologs, Yjl070p and Ybr284p, indicate that these proteins have no detectable AMP-, adenosine- or adenine deaminase activity, but instead we show that overexpression of *YJL070c* mimics a loss of AMPD function. Expression of the yeast transcriptome was monitored in a AMPD deficient mutant, in a strain overexpressing *YJL070c* and in cells treated with the immunosuppressive drug mycophenolic acid, three conditions leading to severe depletion of the guanylic nucleotide pool. These three conditions resulted in the up- or down-regulation of multiple transcripts, 244 of which are common to at least two conditions and 71 to all three conditions. These transcriptome results, combined with specific mutants analysis, point to threonine metabolism as exquisitely sensitive to the purine nucleotide balance.
INTRODUCTION

Purine nucleotides, ATP and GTP, are involved in almost all aspects of cellular life. Besides their role as building blocks of nucleic acids, adenylic and guanylic nucleotides have specific roles. For example, GTP is critical for translation and for signaling through GTPases, while ATP is the major energy providing molecule in the cell. In yeast, intracellular concentrations of ATP and GTP are clearly different (about 5 and 1.5 mM, respectively (BRETON et al. 2008; GAUTHIER et al. 2008)) most probably as the result of regulatory processes maintaining homeostasis. In most eukaryotic cells including yeast, adenylic and guanylic nucleotides are either synthesized from a common precursor (IMP) or recycled from preformed bases or nucleosides (Figure 1). While most enzymes involved in these processes have been identified and studied, the physiological consequences of purine nucleotide imbalance are far from being understood. Interestingly, drugs specifically inhibiting GTP synthesis, such as mycophenolic acid (MPA), have a strong immunosuppressive effect and are now widely used to limit allograft rejection. We have previously established the effect of MPA on the yeast proteome (ESCOBAR-HENRIQUES et al. 2001) and identified numerous yeast mutants hypersensitive to this drug (DESMOUCELLES et al. 2002). These studies suggested that most MPA effects were associated with GTP shortage because these effects were largely reversed by addition of exogenous guanine allowing to replenish the GTP pool via the salvage pathway. However, because drugs often have secondary effects and can be detoxified and/or diluted during cell growth, well controlled physiological studies of their effects are not trivial. We therefore intended to use yeast mutants as a complementary approach to study the consequences of purine nucleotide imbalance. In two previous studies, we have used specific mutants to increase the GTP pool or decrease the ATP pool (BRETON et al. 2008; GAUTHIER et al. 2008). In both cases, we have combined measurement of nucleotide pools with
transcriptome and phenotypic analysis, revealing in both cases specific defects associated with the mutations. In this study, we now take advantage of a conditional phenotype of an AMP deaminase mutant to revisit GTP shortage consequences in yeast.

AMP deaminase (AMPD; EC 3.5.4.6) is an important enzyme for purine interconversion. During muscle effort, extensive hydrolysis of ATP into ADP results in massive AMP production due to adenylate kinase (myokinase) activity. Under such conditions AMPD, by draining AMP to IMP, plays a critical role in the stabilization of adenylate energy charge. Consequently, defects in AMPD lead to exercise-induced muscle symptoms such as early fatigue. Indeed, AMPD defect is the most common muscle enzyme defect in man (FISHBEIN et al. 1978). While there are three AMPD human isoforms, in *Saccharomyces cerevisiae*, a mutation at a single locus (named *AMD1*, Figure 1) abolishes AMPD activity (MEYER et al. 1989). However, two yeast proteins of unknown function (Ybr284p and Yjl070p) are more than 30% identical to Amd1p in their C-terminus (Supplemental Figure 1) (DUENAS et al. 1999; VANDENBOL and PORTETELLE 1999). Strikingly, in most organisms AMPD isoforms have highly divergent N-terminus and in several cases, including yeast, the N-terminus of the protein is not required for activity and is often lost during protein purification due to proteolysis (MERKLER et al. 1989; SABINA and MAHNKE-ZIZELMAN 2000). Yeast AMP deaminase activity has been characterized *in vitro* (MEYER et al. 1989) and is presumed to be highly active under specific growth conditions where massive synthesis of IMP from AMP has been observed (LORET et al. 2007; OSORIO et al. 2003; SILLES et al. 2005). IMP can then be metabolized through three different pairs of enzymatic reactions. It can give back AMP *via* two enzymatic steps encoded by the *ADE12* and *ADE13* genes, it can be transformed to GMP *via* IMP dehydrogenase and GMP synthetase and finally, it can be degraded into inosine and hypoxanthine *via* the successive action of IMP specific nucleotidase (Isn1p) and purine nucleoside phosphorylase (Pnp1p).
Therefore yeast AMP deaminase appears as a critical enzyme for both purine interconversion and degradation. While the biochemical properties of yeast AMP deaminase have been well studied ((MERKLER et al. 1993; MERKLER and SCHRAMM 1990; MERKLER and SCHRAMM 1993; MERKLER et al. 1989)), the physiological consequences of an AMD1 defect have not been investigated and initial reports do not mention any major phenotype associated with the lack of AMP deaminase.

In this paper, we show that under specific conditions, a defect in AMPD activity strongly impairs yeast cell growth and we establish that AMPD plays a crucial role in maintaining guanylic nucleotide homeostasis. Study of the Amd1p homologs, Yjl070p and Ybr284p, revealed that overexpression of these proteins cannot suppress phenotypes associated to amd1 deletion. Instead, we show that overexpression of YJL070c, in a wild-type strain, mimics a loss of Amd1p function. Transcriptome analyses were then performed using these various constructs and MPA treated cells, thus allowing us to evaluate the consequences of guanylic nucleotide depletion obtained through different means. These new genetic tools were also used to revisit the phenotypes of several MPA hypersensitive mutants.
**MATERIAL AND METHODS**

**Yeast Media:** SD minimal medium contains 0.5% ammonium sulfate, 0.17% yeast nitrogen base Difco, 2% glucose. SC was prepared as described (Sherman 1986). SDcasaW is SD medium supplemented with 0.2% casamino acids (Difco) and tryptophan (200 µM). When indicated, adenine, hypoxanthine and guanine were added at 300 µM and uracil was added at 180 µM.

**Yeast Strains:** All strains belong to, or are derived from, a set of disrupted strains isogenic to BY4741 (MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0) or BY4742 (MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0) purchased from Euroscarf. Double, triple and quadruple yeast mutant strains were constructed by mating, sporulation and dissection. The following mutant strains were constructed: Y2077 (MATα, amd1::kanMX4, aah1::kanMX4, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0), Y2362 (MATa, ade8::kanMX4, amd1::kanMX4, aah1::kanMX4, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0), Y2693 (MATa, his1::kanMX4, ade8::kanMX4, amd1::kanMX4, aah1::kanMX4, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0).

**Plasmids:** The IMD2-lacZ (P354, 2µ URA3 and P777, 2µ, LEU2) and the pCM189 (CEN, URA3) plasmids used in this study were previously described (Escobar-Henriques and Daignan-Fornier 2001; Gari et al. 1997). The tet-APT1 (P2091), tet-YJL070c (p2483), tet-HPT1 (P2149), tet-AMD1 (P2479) and tet-YBR284w (P2481) plasmids were obtained by PCR amplification of the corresponding genes using oligonucleotides 527 (5’-CGCGGATCCATCATGTCTATAGCGATTAT-3’) and 528 (5’-CCAATGCATTCATTTTTTCAACGCTTCCTT-3’) for APT1, 973 (5’-CGCGGATCCATTATGCAGCGGCTTAGAGAG-3’) and 974 (5’-CGCGGATCCATTATGAGGCGGTTTAGAGAG-3’).
CAGGTTATGCATCTACCAAATGGAGTTAATTAC-3') for YJL070c, 551 (5'-CGCTGATCAATGTCGGCAAACGATAAGCAA-3') and 552 (5'-AAACTGCAGTGTTTGGTTCCTGCTC-3') for HPT1, 969 (5'-CGCAGATCTATTATGGACAATCAGGCTAC-3') and 970 (5'-CAGGTTCTGCAGTCACGCAATTATGAGTCGCC-3') for AMD1, 971 (5'-CGCGGATCCACAATGGTACAGAACAATGAATC-3') and 972 (5'-CAGGTTCTGCAGTCAGTAATTAGTACAGTCGC-3') for YBR284w. The PCR-amplified fragments were cut with BamHI and NsiI (for APT1 and YJL070c), BclI and PstI (for HPT1), BglII and PstI (for AMD1) and BamHI and PstI (for YBR284w) and were ligated in pCM189 opened with BamHI and PstI.

**β-galactosidase activity**: β-galactosidase (βGal) assays were performed as described (KIPPERT 1995) on cells grown for 6 h in the presence or the absence of purine. βGal units are defined as \([\text{optical density at } 420 \text{ nm } \times 1,000/\text{optical density at } 600 \text{ nm } \times \text{minutes } \times \text{milliliters}]\).

**Transcriptome analysis**: DNA microarray analysis was done to determine the global transcriptional response to amd1 deletion, mycophenolic acid (MPA) treatment and YJL070c overexpression. In these experiments, the amd1 deletion mutant was compared to the isogenic wild-type strain (BY4742); the BY4742 wild-type strain carrying an YJL070c overexpression plasmid was compared to the same strain carrying an empty vector; finally wild-type cells treated or not with mycophenolic acid (0.03 or 0.1 mg/l for 1 hour) were compared. For all comparisons, cells were grown overnight in SDcasaWAU medium, diluted in 50 ml of the same medium and harvested 24 hours latter in exponential phase at A600 = 0.6-0.8. RNA were extracted as described in the Gene Expression Omnibus (GEO) entry for this article and
were purified with RNeasy purification kit (Quiagen) according to the manufacturer’s protocol. cDNA labeling with Cy3 and Cy5 (2 µg of RNA/reverse transcription reaction) and cDNA probing on Agilent DNA microarray slide (GE 8 x 15K n° AMADID 015761) were done as described in GEO. The arrays were read with a Genepix 4000B scanner. Two hybridizations were performed for each comparison using the dye-swap procedure. Normalization was done by the locally weighted scatter plot smoothing (LOWESS) algorithm (BENGTSSON et al. 2004). Complete micro-array raw data sets and experimental details are available in GEO under the GSE9557 accession number.

**Nucleotide pools:** Intracellular nucleotide determination was performed as previously described (BRETON et al. 2008; GAUTHIER et al. 2008).

**Growth test:** Yeast cells were re-suspended in sterile water to an A_{600} (Absorbance at 600 nm) = 1 and submitted to 1/10 serial dilutions. Drops (5 µl) of each dilution were spotted on freshly prepared plates and were incubated at 30°C for 48 h to 72 h.
RESULTS

Deletion of *AMD1* affects growth in the presence of adenine and impairs purine nucleotide balance: While no major growth defect associated with the lack of AMP deaminase activity has been reported previously (Meyer et al. 1989), cautious phenotypic examination of an *amd1* deletion mutant revealed a specific growth defect when adenine is added to the growth medium. In particular, we observed a strong defect in germination and/or growth of *amd1* spores in the presence of adenine (Figure 2A). During vegetative growth, doubling time of the wild-type strain in SDCasa medium (82 min) was not affected by adenine (83 min) while for the isogenic *amd1* mutant, doubling times were 90 min and 140 minutes in the absence and presence of adenine, respectively. This observation prompted us to measure purine nucleotide pools in the wild-type and *amd1* mutant strains grown in the presence or absence of adenine. HPLC measurement revealed that, as expected, adenylic nucleotides were more abundant in the *amd1* mutant which cannot convert AMP to IMP (Figure 2B). This effect was further enhanced by addition of adenine in the growth medium. Second, we observed that both the GDP and GTP pools were severely diminished (3 and 2 folds, respectively) in the *amd1* mutant grown in the presence of adenine compared to the wild-type strain (Figure 2C). In the absence of adenine, GDP and GTP pools were not significantly affected in the *amd1* mutant (Figure 2C). These results establish that Amd1p is critical to maintain the purine nucleotide balance in yeast and suggest that the *amd1* growth defect in the presence of adenine could be due to the inability of this mutant to correctly balance adenylic and guanylic nucleotides. A simple interpretation would be that, in the *amd1* mutant, impaired synthesis of IMP from AMP results in insufficient guanylic nucleotide synthesis.

In the presence of adenine, there are three possible routes to synthesize IMP (and in turn GMP). The first route goes through adenine phosphoribosyltransferase (Apt1p) and AMP
deaminase (Amd1p), the second route goes through adenine deaminase (Aah1p) and hypoxanthine-guanine phosphoribosyltransferase (Hpt1p), and the third route is de novo synthesis from PRPP (Figure 1). In the presence of adenine, this last route is strongly inhibited due to feedback control on the first enzyme of the pathway (Ade4p) and also to transcriptional control on all the genes of the de novo pathway (DAIGNAN-FORNIER and FINK 1992; REBORA et al. 2001). Thus, in the presence of adenine and in the absence of Amd1p, the synthesis of IMP and GMP should mostly take place via Aah1p and Hpt1p and could become limiting for growth. Consistently, the amd1 growth defect was suppressed by overexpression of HPT1 (Figure 3A) which drains adenine toward IMP synthesis. On the other hand, the amd1 growth defect was enhanced by overexpression of APT1 (Figure 3A), which metabolizes adenine to AMP and therefore competes with Aah1p for available adenine (Figure 1). As expected, an aah1 mutant, which blocks the second route, also showed a slight growth defect in the presence of adenine (Figure 3A) and consistently, in the case of aah1, the growth defect was suppressed by overexpression of APT1 (Figure 3A), which metabolizes adenine to AMP, the Amd1p substrate (Figure 1).

We then tested the hypothesis that the amd1 phenotype would be exacerbated by combining it with mutations affecting other IMP supplying enzymes. Based on the results presented above, an enhanced growth defect was expected for an aah1 amd1 double mutant. Indeed, in the presence of adenine, growth of such a double mutant was much more affected than that of each single mutant (Figure 3B). Residual growth in this mutant was due to IMP synthesis via AICAR from the purine and histidine de novo pathways (REBORA et al. 2005) (Figure 1) and could be totally abolished in the ade8 his1 aah1 amd1 quadruple mutant (Figure 3B). As expected, this quadruple mutant, that does not allow conversion of adenylic to guanylic nucleotides, was fully viable when hypoxanthine was provided as a purine source (Figure 3B). Furthermore, growth of the quadruple mutant in the presence of adenine was
restored to a certain extent by addition of guanine, indicating that the growth defect is, at least in part, due to guanylic nucleotide shortage (Figure 3C). Incomplete suppression by guanine could be due to the fact that guanylic nucleotide shortage is not the sole problem faced by this strain. Alternatively, it could be due to poor guanine uptake in the presence of adenine since both compounds are transported by the purine-cytosine permease (SCHMIDT et al. 1984).

**Overexpression of YJL070c phenocopies an amd1 knock-out mutant:** As mentioned in the introduction section, there are two AMD1 homologs named YBR284w and YJL070c, the function of which is unknown. Importantly, both proteins lack several residues (Supplemental Figure 1) conserved in all described purine deaminases (RIBARD et al. 2003). Overexpression of these genes could not rescue growth of the quadruple aah1 ade8 amd1 his1 mutant in the presence of adenine (Supplemental Figure 2A), indicating that, under these conditions, these genes do not significantly contribute to yeast AMPD activity. Of note, we also showed that these genes do not contribute significantly to adenosine- or adenine deaminase activity (Supplemental Figure 2A-B). Finally, mutations in these two genes, either alone or combined with amd1, did not result in additional phenotypes (Supplemental Figure 2C). However, to our surprise, we found that overexpression of YJL070c, but not that of YBR284w, affected growth of a wild-type strain, specifically in the presence of adenine (Figure 4A). This phenotype is reminiscent of the one observed for an amd1 mutant (Figure 2A). Importantly, we found that the effect of YJL070c overexpression was dependent on the presence of Amd1p since YJL070c overexpression had no additional effect in the amd1 deletion mutant (Figure 4A). We thus investigated whether overexpression of YJL070c would mimic other phenotypes of the amd1 knock-out. Indeed, we found that the phenotype, associated to YJL070c overexpression, was strongly enhanced in the aah1 mutant (Figure 4A) as previously found for the amd1 mutation (Figure 3B). Measurement of guanylic nucleotide
pools revealed that overexpression of \textit{YJL070c} resulted in a strong decrease of GDP and GTP intracellular concentration (Figure 4B). This effect was only observed when cells were grown in the presence of adenine as previously found for the \textit{amd1} mutant (Figure 2C). In yeast, a guanylic nucleotide limitation results in a strong induction of the \textit{IMD2} gene expression and therefore, the \textit{IMD2-lacZ} fusion is a convenient reporter to detect deficient GMP synthesis (Escobar-Henriques and Daignan-Fornier 2001). Clearly, overexpression of \textit{YJL070c} resulted in significant induction of \textit{IMD2-lacZ} expression specifically in the presence of adenine (Figure 4C) and consistently, \textit{IMD2-lacZ} expression was also strongly induced in the \textit{amd1} mutant specifically in the presence of adenine while it was not affected in the \textit{ybr284w} and \textit{yjl070c} mutants (Figure 4D). Therefore, according to all tested criteria, overexpression of \textit{YJL070c} phenocopies a \textit{amd1} deletion.

\textbf{Transcriptome analysis of guanylic nucleotide depleted yeast cells:} The \textit{amd1} mutant and the overexpression of \textit{YJL070c} described in the previous sections now provide us with new ways to challenge cells with guanylic nucleotide depletion. We therefore took advantage of these strains to evaluate the effects of GDP and GTP limitation on the yeast transcriptome and to revisit the effects of the immunosuppressive drug MPA which specifically inhibits eukaryotic IMP dehydrogenase (IMPDH). The effect of MPA on yeast purine nucleotide pools was determined for two MPA concentrations. As expected, in MPA treated cells, GDP and GTP concentrations were severely affected in a dose dependent manner (Figure 5A), while MPA treatment only slightly affected adenylic nucleotide pools (Figure 5A). Importantly, MPA treatment also led to massive accumulation of IMP the substrate of IMPDH and its nucleoside (inosine) and base (hypoxanthine) derivatives (Figure 5A). Of note, the \textasciitilde 50\% decrease in GTP concentration found in both the \textit{amd1} mutant and the \textit{YJL070c} overexpressing strains (Figure 2C and 4B) is intermediary to the 17\% and 80\%
found for the lower and higher MPA concentrations, respectively.

The effects of guanylic nucleotide shortage on the yeast transcriptome were then evaluated in a wild-type strain treated or not with MPA (0.03 and 0.1 mg/l), in an *amd1* mutant and in a wild-type strain overexpressing or not *YJL070c*. The first conclusion was that MPA treatment affected expression of multiple genes either up- or down-regulated. The number of affected genes was much higher at 0.1 mg/l MPA compared to 0.03 mg/l (Figure 5B). While most of the 51 genes affected at low MPA concentration are also affected at the higher concentration, 14 (27%) were not, suggesting that there is not a simple transcriptional response to the drug.

Comparison of the transcriptome in *YJL070c* overexpression and *amd1* deletion strains to their cognate wild-type control strains revealed an altered expression for 358 and 407 genes, respectively, 246 of which were affected in both conditions (Figure 5C). This result strongly supports our genetics and biochemical data indicating that overexpression of *YJL070c* phenocopies a *amd1* deletion. A list of all affected genes is presented in supplementary Table 1. When looking at the five most affected genes in each condition (Figure 5D) several conclusions can be drawn. First, as expected, in all four conditions *IMD2* expression was strongly up-regulated (more than 10 fold) thus confirming the *IMD2-lacZ* results (Figure 5D). Second, some genes, such as *SSA4* or *HSP82*, were strongly and specifically affected by MPA, even at the lower dose, but did not respond to GTP shortage induced by *amd1* or *tet-YJL070c*. Because these genes are involved in stress response, this could reflect a by-effect of MPA not directly linked to GTP synthesis inhibition. Reciprocally, genes such as *ICY2* or *LEU4*, strongly responded to the *amd1* mutation and *YJL070c* overexpression but not to MPA treatment. Finally in most cases (*LYS1, ARG1, CPA2, SNZ1, TMT1, HIS4*) the transcriptional response seems gradual, low at the low MPA dose, higher at 0.1mg/l MPA and even higher when *amd1* is mutated or *YJL070c* overexpressed. This could
appear surprising since the high dose of MPA treatment leads to lower GTP concentrations, but could reflect secondary effects of the drug. Interestingly, many of these genes are under control of the transcriptional factor Gcn4p and it should be noticed that GCN4 itself is transcriptionally induced in the amd1 mutant and the tet-YJL070c conditions but is unaffected in the MPA treated cells (Figure 5E). Furthermore, PCL5, a major regulator of Gcn4p stability (Shemer et al. 2002) is also differentially induced (Figure 5E) and could negatively modulate GCN4 overexpression. Global comparison revealed 71 genes similarly affected by amd1 knock-out, YJL070c overexpression and 0.1 mg/l MPA treatment (Supplemental Figure 3), most of which were involved in amino acid metabolism and regulated by Gcn4p (Natarajan et al. 2001). Thus, although GCN4 itself responds differently under the various conditions, GTP shortage is associated with up-regulation of multiple GCN4 target genes.

**Tet-YJL070c as a tool to revisit MPA hypersensitive mutants:** Our earlier search for yeast mutants sensitive to MPA treatment had revealed that eight MPA-sensitive mutants were amino acid metabolism mutants (Desmoucelles et al. 2002). Since our transcriptome analysis revealed strong induction of several such amino acid metabolism genes when guanylic nucleotides are scarce, we used YJL070c to reinvestigate this phenotype. The tet-YJL070c construct was found to severely affect growth of three mutants (tat1, thr1 and hom2) on adenine, it had a weaker effect on the ilv1, bap1, ser2 and hom6 mutants and no major effect on ser1 (Figure 6A). As a control experiment, we showed that a amd1 thr1 double mutant behaved just as the thr1 mutant carrying the tet-YJL070c plasmid, thus confirming once again that overexpressing YJL070c could phenotypically mimic the amd1 deletion (Supplemental Figure 4). Importantly, hom2 and thr1 growth defect, associated to YJL070c overexpression, was suppressed by addition of threonine in large excess to the growth medium (Figure 6B). Since both hom2 and thr1 mutations block threonine biosynthesis, this
result strongly suggests that threonine uptake is limiting for growth in these mutants when guanylic nucleotides are scarce. Thus, this phenotypic analysis supports the idea that there is a connection between guanylic nucleotide limitation and amino acid metabolism.

In yeast, hypersensitivity to MPA is often used as a criterion to identify transcriptional elongation mutants, the rationale being that GTP shortage (substrate limitation) combined to an elongation default affects transcription efficiency and/or accuracy so severely that it results in growth impairment. However, in several of these MPA-sensitive mutants, such as dst1 (ppr2), expression of IMD2, which encodes the MPA resistant isoform of IMPDH (HYLE et al. 2003), is drastically decreased (SHAW and REINES 2000). Thus, there are two major and non exclusive hypotheses to explain why these mutants are hypersensitive to MPA. The first one is that sensitivity to MPA could be due to IMD2 low expression. The second one is that the mutants could be sensitive due to the combination of low GTP concentration and altered transcription elongation. The tet-YJL070c construct, because it allows to decrease GTP concentration independently of MPA, was used to attempt to settle this question.

Consistently with the first hypothesis, expression of IMD2 under an heterologous tet promoter reversed MPA hypersensitivity of the dst1 mutant strain (Figure 7A) and importantly it did not significantly suppress the adenine specific growth defect of the amd1 mutant, albeit it slightly improved growth of all the strains in the presence of adenine (Figure 7B). Thus, although both MPA treatment and amd1 mutation induce IMD2 expression (Figure 4 and Supplemental Figure 3B), the growth defect associated with the amd1 mutation is independent of IMD2 expression. Similarly, IMD2 overexpression had no effect on the growth defect due to overexpression of YJL070c in the presence of adenine (Figure 7C).

To test the second hypothesis and establish whether a decrease of GTP concentration in various transcription mutants was sufficient to explain their hypersensitivity to MPA, we took advantage of the tet-YJL070c plasmid allowing to decrease GTP pools independently of
MPA treatment. We thus overexpressed \textit{YJL070c} in various MPA hypersensitive mutants proposed to affect transcription elongation and monitored growth and GTP concentration. Strikingly, while overexpression of \textit{YJL070c} in the mutants and in the isogenic wild-type strain similarly affected GTP levels (Supplemental Figure 5), it had no drastic effect on growth in the presence of adenine (Figure 7D). Of note, the double \textit{amd1 dst1} mutant behaved just as the \textit{dst1} mutant carrying the \textit{tet-YJL070c} plasmid (Supplemental Figure 4). Together, our results indicate that a significant part of the MPA sensitivity of these mutants is most likely due to poor expression of \textit{IMD2} rather than to a synthetic growth defect due to impaired transcription elongation combined with GTP limitation. However, it cannot be excluded that the growth defect of these transcription mutants observed in the presence of MPA could necessitate a more severe GTP limitation than the one caused by \textit{YJL070c} overexpression in the presence of adenine.
DISCUSSION

A major conclusion from our results is that, in yeast, a defect in AMP deaminase is associated with a severe GDP/GTP pool depletion. This effect could only be observed in the presence of adenine, under conditions where the de novo pathway is switched-off. We interpret this result as follows: in the absence of adenine, IMP synthesized from de novo pathway is sufficient to provide wild-type levels of GDP and GTP. Addition of extracellular adenine results in a strong down-regulation of the de novo pathway (DAIGNAN-FORNIER and FINK 1992) and a subsequent decrease of IMP synthesis which, in the amd1 mutant, cannot be fully compensated for by IMP synthesis via adenine deaminase. Indeed, we found that both the growth defect and the decrease of GDP/GTP pools only occurred in the presence of adenine. Interestingly, a recent report on Arabidopsis thaliana AMP deaminase inhibition by deaminoformycin showed a synergistic effect of adenine on deaminoformycin toxicity (XU et al. 2005). This phenotype is highly similar to our observation showing the inhibitory effect of adenine on growth of the amd1 mutant. However, in A. thaliana, adenylic nucleotide accumulation appears to be the initial cause of growth inhibition; by contrast, in yeast, partial reversion of the adenine effect by guanine (Figure 4B) suggests that guanylic nucleotide shortage is, at least in part, responsible for growth inhibition. In our previous work, we have studied the transcriptional response to ATP limitation (adk1 mutant (GAUTHIER et al. 2008)) and GTP overproduction (constitutive HPT1 mutant (BRETON et al. 2008)). In this work, we now document the transcriptional response to GTP shortage obtained by different means. Importantly, while all these conditions result in purine nucleotide unbalance there is no evidence for a common transcriptional response. Furthermore, GTP shortage and overproduction do not result in opposite transcriptional responses.

In this study, important data were collected on the yeast AMP deaminase gene family. Based on phenotypic analysis, we established that neither of the two AMD1 homologs,
YBR284w and YJL070c, encode AMP-, adenosine- or adenine deaminase activity (supplemental Figure 2). These results are in agreement with the fact that both proteins lack important residues (supplemental Figure 1) conserved in all described purine deaminases (RIBARD et al. 2003). However, because these genes are syntenic with genes in other fungi (S. paradoxus, S. bayanus, A. gossypii...), and since the Yjl070p protein has been detected by mass spectrometry (GHAEMMAGHAMI et al. 2003), it seems unlikely that these genes could be pseudo-genes. Concerning Yjl070p, we favor the hypothesis that it could have a non-catalytic regulatory function. Indeed, we found that overexpression of Yjl070p, in the presence of adenine, has a strong effect on guanylic nucleotide concentration and growth was impaired. These results are highly similar to those obtained with the amd1 deletion, indicating that overexpression of YJL070c mimics the amd1 knock-out. Importantly, Amd1p and Yjl070p co-purified in a global proteome interaction analysis (KROGAN et al. 2006), thus suggesting a possible direct interaction that could affect AMP deaminase activity. An attractive working hypothesis would be that Yjl070p could form inactive heterodimers with Amd1p, however, our attempts to document a direct inhibitory effect in vitro were unsuccessful (C.S.-M. and B.D.-F. unpublished data). The fact that YJL070c overexpression mimicked the amd1 deletion was confirmed by our transcriptome analysis showing that a large set of common genes were induced in both conditions. The transcriptional response to MPA was more divergent. This could be due to the fact that MPA treatment, by inhibiting IMPDH, leads to massive accumulation of inosine (up to 12 mM) as well as IMP and hypoxanthine, although to a lesser extent. No accumulation of these compounds was observed in the amd1 mutant or YJL070c overexpression strains.

We also took advantage of GTP limitation induced by YJL070c overexpression to revisit the proposed phenotypical link between GTP shortage and transcription elongation mutants, which are hypersensitive to MPA. We found that all tested mutants (paf1, dst1, rtf1
and ctk1) were not more affected, than the wild-type control, by YJL070c overexpression. Interestingly, a study of transcription elongation in vivo indicated that dst1, rtf1 and ctk1 mutations, on their own, do not affect transcription elongation or processivity (MASON and STRUHL 2005). We therefore favor the hypothesis that MPA hypersensitivity of these mutants is due to the need for these factors in MPA-induced IMD2 up-regulation and not a secondary effect of low GTP concentrations upon transcription per se. These results cast a doubt upon the use of MPA sensitivity as a criterion to identify transcription elongation mutants.

Our transcriptome data revealed 71 genes affected at least two fold in all three conditions leading to GDP/GTP shortage (Figure 6B). Strikingly, many of these genes are involved in amino acid metabolism and the vast majority of them are under control of the Gcn4p transcription factor. This result is in good agreement with previous reports showing induction of GCN4 target genes in response to severe purine limitation (ROLFES and HINNEBUSCH 1993). An important result is that some amino acid metabolism mutants appear exquisitely sensitive to guanylic nucleotide limitation. We found that threonine biosynthesis mutants, thr1 or hom2, are strongly affected by YJL070c overexpression and that this growth defect could be alleviated by increasing external threonine concentration. Interestingly, thr1 and hom2 mutants are hypersensitive to hydroxyurea (HARTMAN and TIPPERY 2004) and threonine metabolism has been shown to be important for dNTP pool homeostasis in yeast (HARTMAN 2007), however the precise mechanism leading to this cross-pathway effect is not known. In addition, both thr1 and hom2 mutants showed reduced fitness under various growth conditions (GIAEVER et al. 2002), suggesting that threonine availability could be central in responding to different cellular perturbations. Together, our results point to amino-acid uptake as limiting under guanylic nucleotide shortage, thus revealing a new metabolic crosstalk that could be relevant for understanding the immunosuppressive and anti-proliferative effects of mycophenolate derivatives.
ACKNOWLEDGMENTS

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FIGURE LEGENDS

FIGURE 1.—Schematic representation of purine metabolism in *S. cerevisiae*. Abbreviations: Ado: Adenosine; AICAR: 5'-phosphoribosyl-5-amino-4-imidazole carboxamide; Ino: inosine; IMP: Inosine 5'-monophosphate and PRPP: 5-phosphoribosyl-1-pyrophosphate. Gene names are italicized. For simplicity, only the enzymatic steps cited in the text are shown in the figure. Putative adenosine deaminase activity is indicated by a question mark.

FIGURE 2.—External adenine affects growth and nucleotide pools of AMP deaminase deficient strains. (A) Growth of wild-type and *amd1* spores in the absence or the presence of adenine. A heterozygous *AMD1/amd1::KanMX4* diploid strain was sporulated and dissected on SDcasaWU medium containing adenine (+Ade, tetrads 4-6) or not (-Ade, tetrads 1-3). “+” and “−” signs above the colonies refer respectively to *AMD1* (+) and *amd1::KanMX4* (−) genotypes determined on the basis of sensitivity or resistance to geneticin associated with KanMX4 expression. (B-C). Intracellular nucleotide content is affected in the *amd1* mutant grown in the presence of extracellular adenine. Wild-type (WT, BY4742) and *amd1* mutant strains were grown in SDcasaW medium supplemented (+Ade) or not (-Ade) with external adenine. Internal adenylic (B) and guanylic (C) nucleotides were measured as previously described (BRETON *et al.* 2008; GAUTHIER *et al.* 2008).

FIGURE 3.—The growth defect of the *amd1* mutant is enhanced by mutations affecting IMP synthesis and partially suppressed by guanine addition. (A) Overexpression of adenine phosphoribosyl transferase exacerbates the growth defect of the *amd1* mutant in the presence of external adenine. Cells were transformed with the pCM189 plasmid (vector) or plasmids allowing overexpression of adenine phosphoribosyl transferase (*tet-APTI*) or hypoxanthine
guanine phosphoribosyl transferase (tet-HPT1). Transformants in exponential growth phase were serial diluted and spotted on SDcasaW medium supplemented with adenine (+Ade) or hypoxanthine (+Hyp). (B) Combinations of the amd1 mutation with aah1, ade8 and his1 lead to drastic decrease of growth in the presence of adenine. (C) Growth defect of the amd1 aah1 ade8 his1 mutant in the presence of adenine is due to guanylic nucleotide starvation. The quadruple amd1 aah1 ade8 his1 mutant was transformed with the pCM189 empty plasmid (vector) or the plasmid allowing overexpression of AMD1 (p2479). Serial dilutions of yeast cells were spotted on SDcasaW medium supplemented with adenine (+Ade), guanine (+Gua), or hypoxanthine (+Hyp), as indicated.

**FIGURE 4.**—Overexpression of YJL070c phenocopies the amd1 deletion mutant. (A) Effect of YJL070c or YBR284w overexpression on growth in the presence of adenine or hypoxanthine. Wild-type (WT, BY4742), amd1 and aah1 strains were transformed with the pCM189 empty plasmid (vector) or either the tet-YBR284w or tet-YJL070c plasmids. (B) Intracellular guanylic nucleotide content is specifically decreased in wild-type cells overexpressing YJL070c and grown in the presence of adenine. Wild-type (BY4742) cells were transformed with the pCM189 vector or plasmids allowing overexpression of AMD1 (p2479), YJL070c (p2483) or YBR284w (p2481). Transformants were grown in SDcasaW medium supplemented or not with external adenine and intracellular guanylic nucleotide content was measured as previously described (BRETON et al. 2008). (C) Overexpression of YJL070c leads to derepression of the IMD2-LacZ fusion expression only in the presence of adenine. Cells were co-transformed with IMD2-lacZ plasmid (p777) and either the vector or the plasmids allowing overexpression of AMD1 (p2479) or YJL070c (p2483). Transformants were grown in SC medium lacking uracil and leucine and supplemented or not with adenine (+A), guanine (+G) or hypoxanthine (+H). D. Expression of IMD2-LacZ fusion is drastically increased in an
*amd1* mutant in the presence of adenine. Cells were transformed with a plasmid carrying a *IMD2-lacZ* fusion (p354). Transformants were grown in SDcasaW medium supplemented or not with adenine (+A), guanine (+G) or hypoxanthine (+H).

**FIGURE 5.**— Global transcriptional response to *amd1* deletion, *YJL070c* overexpression and MPA treatment in the presence of adenine. (A) Intracellular nucleotide content is affected by MPA treatment. Wild-type cells were grown in SDcasaWU medium supplemented with adenine and internal adenylic and guanylic nucleotides were measured as previously described (BRETON et al. 2008; GAUTHIER et al. 2008). (B-C) Transcriptional response to guanylic nucleotide depletion obtained by MPA treatment, *amd1* deletion and *YJL070c* overexpression. Transcriptional response was monitored by microarray analyses as described in material and methods. Venn diagrams show numbers of genes differentially expressed (by a factor 2 or more compared to untreated wild-type) in response to various MPA concentrations (B), in an *amd1* mutant and a strain overexpressing *YJL070c* (C). (D) Expression ratios of the five most up-regulated genes for each comparison set used in the transcriptome analysis. (E) Expression of *GCN4* and *PCL5* in the various conditions.

**FIGURE 6.—** *YJL070c* overexpression severely affects growth of threonine biosynthesis mutants in the presence of external adenine. Cells were transformed with pCM189 (vector) or the tet-*YJL070c* plasmid. Transformants were serially diluted and were spotted on SDcasaW medium supplemented (+ Ade) or not (- Ade) with adenine and threonine (+ Thr) as indicated.

**FIGURE 7.—** Effect of guanylic nucleotide limitation on transcription mutants. Cells were transformed with pCM189 (vector) or the tet-*IMD2* plasmid and transformants were serially diluted and were spotted on SDcasaW medium supplemented or not with MPA (10 mg/l) (A)
or with adenine (Ade) as indicated (B). (C) Cells containing the tet-YJL070c overexpression plasmid were transformed with pCM189 (vector) or the tet-IMD2 plasmid and transformants were serially diluted and spotted on SC medium lacking uracil and leucine and supplemented or not with adenine. (D) Cells were transformed with pCM189 (vector) or the tet-YJL070c plasmid and transformants were serially diluted and spotted on SDcasaW medium supplemented or not with adenine.
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Fig. 1
A

- Ade  + Ade
1  +  -  +  -
2  -  +  -  +
3  -  -  +  -

B

- Ade  + Ade
WT  - Ade  + Ade
  amd1  - Ade  + Ade

C

- Ade  + Ade
WT  - Ade  + Ade
  amd1  - Ade  + Ade

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Fig. 2
Fig. 3
Figure 4

A

B

C

D

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Fig. 4
Intracellular concentration (mM)

YJL070c overexpression (358 genes)
- 232 up
- 14 down

amd1 deletion (407 genes)
- 150 up
- 11 down

MPA (0.03 mg/l) (50 genes)
- 22 up
- 15 down

MPA (0.1 mg/l) (670 genes)
- 283 up
- 351 down

Expression ratios

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Fig.5
A

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Fig.7