Carbon Catabolite Repression in Yeast is Not Limited to Glucose

Kobi Simpson-Lavy & Martin Kupiec

Cells adapt their gene expression and their metabolism in response to a changing environment. Glucose represses expression of genes involved in the catabolism of other carbon sources in a process known as (carbon) catabolite repression. However, the relationships between “poor” carbon sources is less characterized. Here we show that in addition to the well-characterized glucose (and galactose) repression of ADH2 (alcohol dehydrogenase 2, required for efficient utilization of ethanol as a carbon source), ADH2 expression is also inhibited by acetate which is produced during ethanol catabolism. Thus, repressive regulation of gene expression occurs also between “poor” carbon sources. Acetate repression of ADH2 expression is via Haa1, independently from the well-characterized mechanism of AMPK (Snf1) activation of Adr1. The response to extracellular acetate is attenuated when all three acetate transporters (Ady2, Fps1 and Jen1) are deleted, but these deletions do not affect the acetate response resulting from growth with glucose or ethanol as the carbon source. Furthermore, genetic manipulation of the ethanol catabolic pathway affects this response. Together, our results show that acetate is sensed intracellularly and that a hierarchical control of carbon sources exists even for “poor” carbon sources.

Cells have evolved to respond appropriately to changes to both their intracellular and extracellular environments via multiple sensing mechanisms. Some environmental signals [such as osmotic stress] or nutrients [such as glucose] are sensed at the plasma membrane, whereas others [such as nitrogen, or reactive oxygen species] are sensed intracellularly, despite the drawback that damage might be inflicted before the cell can mount its response. In many studied organisms, glucose is the preferred carbon source. In yeasts, multicellular fungi, bacteria, and metazoa the presence of glucose in the environment prevents the utilization of other available carbon sources, by mechanisms known by the general title of “catabolite repression.” In the yeast Saccharomyces cerevisiae, when glucose is present in the extracellular medium, uptake and catabolism of other carbon sources is repressed via three signaling pathways; inhibition of AMPK (Snf1) activation of Adr1. The response to extracellular acetate is attenuated when all three acetate transporters (Ady2, Fps1 and Jen1) are deleted, but these deletions do not affect the acetate response resulting from growth with glucose or ethanol as the carbon source. Furthermore, genetic manipulation of the ethanol catabolic pathway affects this response. Together, our results show that acetate is sensed intracellularly and that a hierarchical control of carbon sources exists even for “poor” carbon sources.

Acetate is of interest to oenologists, both as a factor affecting wine spoilage and as a source of aroma compounds, such as acetate-esters. Alterations in gene expression of the ethanol-acetate pathway have been previously shown to affect acetate acid production during fermentation. However, acetate is also toxic to cells, resulting in programmed cell death and a reduction in chronological life span. This has led to its use as a food preservative, but is of concern to the biofuel industry. Ethanol production from lignocellulose is limited by the accumulation of acetic and other weak organic acids. A search for genes that can reduce this toxicity found that overexpression of the Haa1 transcription factor results in resistance to acetic acid and increased ethanol yield, by mediating increased expression of target genes. Indeed, Haa1 is responsible for the activation of ~80% of the genes that respond to acetic acid, despite the fact that only about half of these genes have the Haa1 binding site in their promoters.

Acetate is actively transported into the cell through the main transporters Jen1 and Ady2, which are subject to strong glucose repression and inhibition of activity. In addition, the undissociated acid undergoes passive/facilitated diffusion through the Fps1 aquaglyceroporin. Therefore, in glucose-containing media Fps1 is the only route by which acetic acid enters the cell, and deletion of Fps1 results in acetic acid resistance. Although Ady2 is the major importer of acetate, deletion of ADY2 does not affect the response to acetate.

In addition to being an environmental resource, acetate is also a metabolic product formed during fermentation and ethanol catabolism in a pathway conserved across eukaryotes. There are five ADH genes in the S.
cerevisiae genome. Cells expressing only ADH1 produce ethanol during fermentation of glucose comparably to wild-type cells, and can metabolise ethanol14. Adh2 has a 20-fold higher affinity for ethanol than Adh135 and thus cells expressing only ADH2 do not produce ethanol during glucose fermentation, but are capable of ethanol catabolism.34 The mitochondrial Ald3 is expressed at low levels and forms the mitochondrial component of the ethanol-acetyldehyde shuttle to regenerate mitochondrial NAD from NADH. However, cells expressing only ADH3 are capable of both produce and utilize ethanol (similarly to ADH1). Cells expressing solely ADH4 or ADH5 are incapable of producing or metabolizing ethanol34. Acetyldehyde is oxidized to acetic acid primarily by the cytoplasmic Ald6 and the mitochondrial Ald4 and Ald5 enzymes; the first two use NADP as a co-factor, whereas the latter utilizes NAD57,38. Acetate is then conjugated to coenzyme-A in the cytoplasm by two acetyl-coA synthetases: Acs1, which is glucose-repressed39 and Acs2, which is constitutively expressed40. Cytoplasmic Acetyl-coA has important roles in fatty acid synthesis41, acetylation of proteins such as histones42, biosynthesis of steroids and amino acids, and for entry into the Krebs’ cycle via the glyoxylate pathway, which is repressed in the presence of glucose43. A simplified diagram of metabolic pathways pertaining to ethanol and acetate metabolism is presented in Fig. S1.

Despite the central role played by acetate in yeast metabolism, the location of acetic acid sensing has not been yet determined. Here we demonstrate that acetate, a product of both fermentation and ethanol catabolism and itself a carbon source, inhibits expression of genes involved in ethanol catabolism. We show that acetate is sensed intracellularly, and a consequence of this is that ethanol metabolism results in the induction of the acetate response. Our results uncover the existence of catabolite repression among sugars that are considered “poor” carbon sources.

**Results**

**Acetate repression expression of genes involved in ethanol metabolism.** Expression of the ADH2 gene, encoding alcohol dehydrogenase, is tightly regulated by carbon source, being low in the presence of glucose, and high on poor carbon sources. Although the inhibition of ADH2 expression by glucose via the inactivation of Snf1 has been well-characterized (Snf1 is needed to activate the Adr1 and Cat6 transcription factors)34, it is still unclear whether ADH2 expression requires a positive signal from the poor carbon source. We therefore examined ADH2 expression in response to a range of carbon sources, by measuring an increase in ADH2β expression requires a positive signal from the poor carbon source. We therefore examined ADH2 expression in response to a range of carbon sources, by measuring an increase in ADH2β expression in response to acetate (Fig. 1c). Following transfer of cells bearing an expression in response to a range of carbon sources, by measuring an increase in ADH2β expression requires a positive signal from the poor carbon source. We therefore examined ADH2 expression in response to acetate (Fig. 1c). Following transfer of cells bearing an expression in response to acetate (Fig. 1c). Following transfer of cells bearing an expression in response to acetate (Fig. 1c). Following transfer of cells bearing an expression in response to acetate (Fig. 1c). Following transfer of cells bearing an expression in response to acetate.
conclude that the Adr1 and Haa1 pathways that regulate ADH2 expression seem to be independent from each other.

**Location of Acetate sensing.** Anionic acetate is imported into the cell by the Jen1 and Ady2 transporters, and at low pH (below 4.76) undissociated acetic acid undergoes facilitated diffusion by Fps1 (reviewed in21). Acetate, being a small molecule can also passively cross the plasma membrane32. However, whether acetate is sensed inside or outside the cell has not been established. We generated double and triple knockouts of the acetate importers, and found that ADH2 expression was restored to 50% of its ethanol expression levels when cells were grown in acetate media upon deletion of all three importers (Ady2, Fps1 and Jen1 - ΔΔΔafj) (Fig. 3a). To confirm that this is due to acetate, we deleted ACS1, which converts acetate to acetyl-coA (Fig. S1) in order to lower the flux from acetate to acetyl-coA. Deletion of ACS1 lowered ADH2 expression and suppressed the high ADH2 expression (in acetate) phenotype of ΔΔΔafj cells (Fig. 3a). We wondered whether the changes of ADH2 expression affect ADH activity for the oxidation of alcohols. We performed ADH assays on yeast extracts from cells grown with different carbon sources. The assay does not discriminate between different ADH enzymes, and so glucose-grown cells have an ADH activity of 20 mU/OD which is present in Δadh2 cells (and is thus due to the other ADH enzymes, primarily Adh1). ADH enzyme activity correlates with ADH2 gene expression above this threshold in ethanol and acetate grown cells, with Δhaa1 and ΔΔΔafj cells having much higher ADH activity in ethanol and acetate conditions which is reversed by additional deletion of ACS1. Interestingly growth with oleate as the sole carbon source results in abrogation of all ADH activity, suggesting post-transcriptional regulation is occurring. Expression of YRO2 was reduced by 50% in the ΔΔΔafj strain) when acetate was added to cells for a 3 hour period (Fig. 3c). We examined the expression of YRO2 in cells grown with differing concentrations of acetate and other carbon sources. Ethanol catabolism produces intracellular acetate, and this was sufficient to induce an acetate response (Fig. 3d). In contrast, oleic acid catabolism does not produce acetate, and YRO2 was not expressed upon growth with oleic acid as the sole carbon source. For determining the effect of acetate concentration upon YRO2 expression, experiments were carried out under respiratory conditions with glycerol as the
carbon source, since glycerol metabolism does not involve \textit{ADH2}. For both BY4741a and W303-1b backgrounds we observed a progressive increase in \textit{YRO2} expression with increasing acetate concentration, though high levels of \textit{YRO2} expression occur only at 1% or higher concentrations of acetate (Fig. 3d). We note that these acetate concentrations are higher than previously reported for \textit{YRO2} expression\textsuperscript{51} since most experiments are carried out in the presence of glucose, which causes acute acetic acid stress\textsuperscript{21}. Similarly to \textit{ADH2} repression (Fig. 1d), the V5 background exhibits an attenuated response to acetate.

To further confirm the importance of the acetate transporters for the acetate genetic response, we examined Haa1-GFP localization in glucose and following addition of acetate (pH 6) to 2% for 45 minutes. As previously reported, Haa1-GFP is constitutively nuclear in \textit{Δhrr25} cells\textsuperscript{51}. Whereas a strong nuclear Haa1-GFP localization is present in WT cells 45 minutes after addition of acetate to 2%, Haa1-GFP did not localize to the nucleus in \textit{ΔΔΔafj} cells (Fig. 3d,e).

Since ethanol catabolism produces acetate, we reasoned that measuring \textit{YRO2} expression (acetate-induced) in cells growing on ethanol versus acetate may provide a reasonable proxy from endogenous versus exogenous acetate. We grew cells over a 24 hour period and measured \textit{YRO2} expression (Fig. 3g). Glucose induces about 15% of \textit{YRO2} expression compared to acetate, presumably due to conversion of acetylaldehyde to acetate during fermentation. Likewise, ethanol induces 40% of the \textit{YRO2} expression seen in acetate. These results imply that conversion of ethanol to acetate is faster than utilization of acetate and that acetate can function as a carbon reserve for cells. In contrast, oleic acid does not induce \textit{YRO2} expression. Whereas \textit{YRO2} expression was reduced by 70% in the \textit{ΔΔΔafj} strain during growth on acetate, expression was unaffected when cells were grown on glucose or ethanol, strongly suggesting that acetate is indeed sensed intra-cellularly. Upon deletion of \textit{ACS1} in \textit{ΔΔΔafj} cells (thus restricting conversion of acetate to acetyl-coA), \textit{YRO2} expression was somewhat increased when cells were grown on acetate (though not to the same extent as in \textit{Δacs1} cells). Furthermore, deletion of \textit{ACS1} also increased \textit{YRO2} expression in glucose and ethanol grown cells, presumably due to increased acetate concentration in the cell (Fig. 3h).

**Other ethanol metabolic genes are also acetate-repressed.** We analyzed expression of the other genes in the ethanol to acetyl-coA pathway (Fig. S1) upon growth on glucose, ethanol, acetate or oleic acid as the sole carbon source. Oleic acid was included as its catabolism provides an alternate source of cytoplasmic acetyl-coA, but does not induce Haa1-mediated transcriptional regulation (which responds to short chain organic acids such as acetic, propionic and lactic acids)\textsuperscript{49}. Whereas \textit{ALD5} expression was unchanged by deletion of \textit{HAA1}, deletion of \textit{HAA1} resulted in modest increases of \textit{ALD4} and \textit{ALD6} expression in acetate and oleic acid media, suggesting that Haa1 contributes to repression of these genes when cells can generate cytoplasmic acetyl-coA (Fig. S2a–c). \textit{ACS1} expression was glucose-repressed as previously reported\textsuperscript{39} and expression in acetate was partially lowered in \textit{Δhaa1} cells (Fig. S2d). \textit{ACS2} expression was strongest in ethanol. No effect of Haa1 could be discerned (Fig. S2e). Thus, it seems that although Haa1 does regulate other genes in the ethanol-acetyl-coA pathway to promote a lowering of acetate levels, the major regulation that Haa1 exerts is at the \textit{ADH2} level.
Metabolic engineering of the acetate response.  

If acetate synthesized intracellularly by ethanol catabolism can be sensed to cause YRO2 expression, then we would expect genetic manipulation of this pathway to exert effects upon gene expression (Fig. 4). Indeed, overexpression of ALD6 results in a dramatic increase in YRO2 expression when cells are grown on glucose or ethanol, but not when grown on acetate or oleate, suggestive of increased acetate production from acetaldehyde. Similarly, YRO2 expression was reduced in glucose- or ethanol-containing media in Δald4 Δald6 cells, which lack two of the five redundant enzymes that convert acetaldehyde into acetate (YRO2 expression in response to acetate was unchanged in Δald4 Δald6 cells.) YRO2 expression in glucose or ethanol was restored in Δald4 Δald6 Δacs1 cells (Fig. 5a). We compared the contribution of ALD4, ALD5 and ALD6. Single deletions alone did not affect YRO2 expression, nor did the Δald4 Δald5 double deletion. Deletion of ALD6 in combination with either Δald4 or Δald5 dramatically reduced YRO2 expression in glucose or ethanol media, and a triple Δald4 Δald5 Δald6 strain eliminates YRO2 expression to the background levels observed in oleic acid (Fig. 5b).
Mutations that increase acetate production (ALD6 overexpression) or reduce its consumption (Δacs1) lower ADH2 expression levels (Fig. 5c). ADH2 expression was elevated in Δald6 cells when cells were grown on 2% ethanol (Fig. 5d), but progressively declined upon further deletion of ALD genes. As expected, ADH2 expression remained low when cells were grown in acetate medium. We suspect that this is due to another layer of regulation of ADH2 expression by the very toxic intermediate acetylaldehyde induced by the restriction of acetyldehyde outflow to acetate.

**Discussion**

Carbon catabolite repression is often considered to be a matter of “good” carbon sources, such as glucose and fructose, actively preventing the metabolism of other carbon sources, and has been much investigated. However, the relationships between poor carbon sources has been of scant enquiry, and thus it was unclear whether similar inter-sugar relationships exist for them, similarly to the relation between glucose and other sugars. Here we have shown that part of the acetate response involves downregulating of ADH2 expression. We also show that there is no positive signal needed to promote ADH2 expression; rather, its expression is negatively regulated by glucose (via Snf1 inhibition of Adr1)⁴⁴, by galactose⁵³ and its downstream product acetate via the Haa1 transcription factor acting as a repressor (Fig. 4). Similarly to glucose, acetate represses ADH2 expression even when other carbon sources are present, illustrating the importance of acetate as a regulatory signal in yeast metabolism.
sources are present. Although gene expression in response to acetate has been previously investigated\textsuperscript{28,29}, this level of regulation has been overlooked since glucose independently inhibits ADH2 expression. In addition, most studies involving acetate provide acetic acid as a non-metabolizable (due to glucose) stressor (pH 4), with both acid and acetate contributing to the stress response. Here we have provided dissociated acetate (pH6) as the sole carbon source. Extracellular acetate (pH6) or intracellularly produced acetate produced via catabolism of glucose or ethanol provoked the Haa1 dependent response previously demonstrated for acetic acid\textsuperscript{30,31}, thus showing that the response is not due to protons but rather is due to the acetate moiety. The intracellular acetate sensor remains to be identified.

We found that ADH activity attributable to ADH2 correlates with ADH2 expression in cells grown with ethanol or acetate as their sole carbon source, although all ADH activity was attenuated in oleic acid grown cells, suggesting further post-transcriptional regulation to occur. It is possible that signaling leading to post translational modification and inhibition of the ADH enzymes may arise from the cleavage and processing of Mga2/Spt23\textsuperscript{54}, or as a consequence of changes to membrane fluid dynamics activating sensors such as Mid1 and Mid2\textsuperscript{55,56}. Although oleic acid does not affect ADH2 or YRO2 expression, it is probable that other inhibitory relationships exist between other carbon sources, which in most studies are masked by an overall glucose repression. However, under conditions where glucose becomes limiting, such mechanisms of metabolic repression are likely to be of significance.

We then utilized the gene expression of ADH2 and YRO2 to determine whether acetate is sensed extracellularly, like glucose or osmotic stress, or whether acetate needs to enter the cell in order to elicit a response. Deletion of all three acetate transporters was required to restore ADH2 expression in acetate, and Haa1-GFP did not localize to the nucleus, nor was YRO2 expressed, in strains lacking these transporters when exposed to acetate, although slowing the efflux of acetate to acetyl-CoA by deletion of ACS1 did partially restore YRO2 expression.

Since acetate is sensed intracellularly, we reasoned that metabolically produced acetate would also elicit an acetate response, albeit to a lesser degree than upon growth with acetate as the sole carbon source. Indeed, the expression of ADH2 or YRO2 was unaffected in a strain deleted for the acetate transporters when cells were grown on either glucose or ethanol as their sole carbon source, further confirming that the effects in acetate media of these deletions is due to a lack of acetate uptake. We further found that mutations in the metabolic pathway that increase acetate production or lower its utilization increase the acetate response, whereas mutations that lower conversion of acetaldehyde to acetate lower the acetate response, the major enzyme responsible being ALD6.

Strikingly, all the metabolites involved in this pathway are toxic\textsuperscript{21,22,52,57,58}, and yet acetate is produced faster than is consumed (thus leading to the acetate response in glucose and ethanol grown cells). However, the consequences of upregulating acetyl-CoA production may not be benign, as cytoplasmic acetyl-CoA is a regulator of autophagy\textsuperscript{59} and directly correlates to histone acetylation levels\textsuperscript{60}. Therefore the yeast response seems to be not to promote acetate metabolism, but rather to deal with the resultant stress\textsuperscript{27,49,61,62}, as shown here, to reduce expression of ADH2. Together, our results uncover a mechanism by which acetate inhibits its own production, resulting in homeostasis of acetate levels (Fig. 4).

**Materials and Methods**

Strains used are listed in Table S1; plasmids used are listed in Table S2. All strains are related to BY4741\textsuperscript{63} except for W303-1b and V5. Standard yeast molecular biology techniques were used for yeast manipulations. To over-express ADL6, ADL6-GFP was inserted into ycpADH1\textsuperscript{64} digested with Sall/SpeI together with the Cyc1 terminator from pUG34. To make the ACS2-LacZ reporter plasmid, the ZWF1 promoter was excised from a plasmid containing prZWF1::LacZ in Ycp50\textsuperscript{65} by BamHI/HindIII digestion, and replaced with 1000 bp of the ACS2 promoter by gap repair.

Media was prepared with 8 g/litre YNB, with 0.0286 g/liter adenine, tryptophan, histidine, arginine, methionine, 0.0714 g/liter phenylalalanine, 0.0857 g/liter tyrosine, lysine, 0.114 g/liter isoleucine, 0.143 g/liter glutamate, aspartate, 0.214 g/liter valine, 0.286 g/liter threonine, 0.571 g/liter serine to make synthetic –LU media. Standard carbon source. Extracellular acetate (pH 6) or intracellularly produced acetate produced via catabolism of glucose or ethanol provoked the Haa1 dependent response previously demonstrated for acetic acid\textsuperscript{30,31}, thus showing that the response is not due to protons but rather is due to the acetate moiety. The intracellular acetate sensor remains to be identified.

For ADH2 induction assays, cells were grown in 4% glucose overnight to ensure complete repression of ADH2 expression, diluted in the morning and grown for an additional 3 hours (t = 0), washed 3x with water, and resuspended in media containing indicated carbon sources for a further 3 hours (t = 3). The data presented is the t = 3 – t = 0/3 and normalized to the WT ethanol sample from that experiment. Typical ADH2 expression was 1500 Miller Units per hour. For YRO2 and other gene expression assays, cells were taken from glucose plates and grown in 3 ml of indicated media for 24 hours without dilution, to prevent loss of secreted metabolites from the medium. Cells visibly grew during this period.

**β-galactosidase assays.** β-galactosidase assays were performed using log phase cells. Cell concentration was determined by reading 80 μl of cells at 595 nm. 20 μl of cells were added to the β-galactosidase reaction mix (40 μl YPER (Ferriec 78990), 80 μl Z-buffer (120 mM Na2HPO4, 80 mM NaH2PO4, 20 mM KCl, 2 mM MgSO4), 24 μl ONPG (4 mg/ml), 0.4 μl β-mercaptoethanol) and incubated at 30 °C for 10 minutes for ADH2, 25 minutes for YRO2 and for 15 minutes for other genes. Reactions were stopped by addition of 56 μl 1 M Na2CO3. The eppendorf tubes were centrifuged for 1 minute at full-speed to pellet the cell debris, and 200 μl supernatant was removed and absorbance read at 420 nm by a microplate reader. Miller Units were calculated by the equation Miller Units = (1000*A420)/(time*0.02*A595 – 0.055, where the A420 and A595 has been corrected for blanking and
path length (final path length = 1 cm). Three biological replicates were used. Error bars are ± 1 standard deviation.

Microscopy. 5µl of log phase cells were imaged using an EVOS microscope (60x objective) with the GFP filter for GFP and the Texas Red filter for Cherry. The dimensions of each panel corresponds to 20 µm × 20 µm. Cells were not concentrated before imaging, to prevent perturbation to their environment. Images were processed using the brightness/contrast function of Image J, to give a black background. For statistics, over 200 cells were counted. Experiments were repeated at least three times on different days. All figures shown in the manuscript are of identical magnification, a size bar is provided in Fig. 3a.

ADH assays. Cells were grown overnight in their respective media. 0.8OD₆₀₀ was harvested and vortexed with glass beads for 20 minutes at 4°C with 100 µl PBS + 0.1% Triton X-100 with Protease (Roche) and Phosphatase (Pierce) inhibitors. Supernatant was centrifuged at 4°C for 10 minutes at 13000 g. An ADH assay kit (Sigma MAK053) was used to determine ADH activity – all volumes were halved from the values suggested in the datasheet, and the amount of developer reduced by half again. Reactions were rapid and completed within 5 minutes. An adhδΔ strain was included as a control. ADH activity is shown as milliUnits per OD₆₀₀ of yeast.

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Author Contributions
K.S.-L. conceived and carried out all experiments, and wrote the paper. M.K. supervised, wrote and revised the paper.

Additional Information
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