Cytosolic Localization of Acetohydroxyacid Synthase Ilv2 and Its Impact on Diacetyl Formation during Beer Fermentation

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Diacetyl (2,3-butanedione) imparts an unpleasant “butterscotch-like” flavor to alcoholic beverages such as beer, and therefore its concentration needs to be reduced below the sensory threshold before packaging. We examined the mechanisms that lead to highly elevated diacetyl formation in petite mutants of Saccharomyces cerevisiae during beer fermentations. We present evidence that elevated diacetyl formation is tightly connected to the mitochondrial import of acetohydroxyacid synthase (Ilv2), the key enzyme in the production of diacetyl. Our data suggest that accumulation of the matrix-targeted Ilv2 preprotein in the cytosol is responsible for the observed high diacetyl levels. We could show that the Ilv2 preprotein accumulates in the cytosol of petite yeasts. Furthermore, expression of an Ilv2 variant that lacks the N-terminal mitochondrial targeting sequence and thus cannot be imported into mitochondria led to highly elevated diacetyl levels comparable to a petite strain. We further show that expression of a mutant allele of the γ-subunit of the F₁-ATPase (ATP3-5) could be an attractive way to reduce diacetyl formation by petite strains.

Apart from the raw materials used, the flavor and taste of alcoholic beverages is strongly affected by fermentation by-products generated during yeast alcoholic fermentation. Although some compounds contribute to a positive sensory impression, others have a negative impact on the flavor and taste of alcoholic beverages. One of these undesirable compounds, diacetyl (2,3-butanedione), imparts an unpleasant “butterscotch-like” flavor to beer and its concentration needs to be reduced to below the taste threshold prior to filtration and packaging. Yeasts produce diacetyl especially in the early phase of fermentation and reduce it later during beer maturation into sensory more neutral compounds such as acetoin and 2,3-butanediol.

The enzyme acetohydroxyacid synthase (Ilv2) is of central importance for diacetyl formation. It catalyzes the first step in the biosynthesis of branched-chain amino acids, the irreversible decarboxylation of pyruvate and the condensation with a second molecule of pyruvate or 2-ketobutyrate. The vicinal diketones diacetyl and pentanedione are formed from these precursors by nonenzymatic oxidative decarboxylation.

The biosynthesis of branched-chain amino acids takes place in the mitochondrial matrix. The nuclear encoded Ilv2 protein is synthesized in the cytosol as a precursor protein with an N-terminal mitochondrial targeting sequence (MTS), which directs import into the mitochondrial matrix. It has been reported that petite mutants of Saccharomyces cerevisiae produce highly elevated levels of diacetyl. Petite mutants have lost part (rho−) or all (rho0) of their mitochondrial genome (mtDNA). Since four subunits of the ADP/ATP carrier (AAC) are encoded in the mitochondrial DNA, the membrane potential is maintained through the exchange of ADP3− for ATP4+. How this cytosolic accumulation of Ilv2 is the reason for the elevated diacetyl production of petite mutants.

**MATERIALS AND METHODS**

Yeast strains, plasmids, and media. The yeast strains used are listed in Table 1. All yeast strains are derived form the CEN.PK background. Insertions and deletions were introduced into the yeast genome by one-step gene replacement with PCR-generated cassettes. The insertions and deletions were verified by PCR. To construct pRK1107, the ATP3 gene (positions 630 to 1009, ATG = +1) was amplified by PCR from yeast chromosomal DNA and cloned into YCplac33. To generate the ATP3-5 mutation (T297A), pRK1107 was mutagenized by QuickChange mutagenesis to give plasmid pRK1108. All cloned genes were sequenced to ensure that no PCR mutations occurred. For standard experiments, yeast cells were grown in synthetic defined medium (1% yeast extract, 2% Bacto peptone, and 2% glucose) or in SD/CAS medium (0.67% yeast nitrogen base without amino acids, 1% casitone hydrolysate, and 2% glucose).
TABLE 1. Yeast strains

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<th>Strain</th>
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<td>MATa his3-Δ1 ura3-52</td>
<td>M. Ramezani-Rad, Düsseldorf, Germany</td>
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RESULTS

Enhanced diacetyl production in petite mutants. It has been reported that respiratory-deficient petite (rho−) mutants accumulate higher diacetyl levels (4), which constitutes a major problem in beer fermentations. This effect could be reproduced in our yeast CEN.PK strain background. Laboratory-scale fermentations were carried out at 30°C with beer wort at cell densities typical of beer fermentations. Samples were collected at time intervals and the diacetyl levels in the samples were determined by headspace gas chromatography with an electron capture detector. For the diploid wild-type strain, very low diacetyl levels were observed with a peak at 8 h after the start of fermentation (0.126 mg/liter) (Fig. 1A). In contrast, diacetyl levels were much higher in the isogenic petite mutant, again with a peak at 8 h of fermentation (0.847 mg/liter). In addition, the sugar profiles and ethanol formation were determined during the course of fermentation. The profiles were

FIG. 1. Diacetyl formation in wild-type and petite strains. Beer fermentations were performed in MEX medium at 30°C for the times indicated. (A to C) Diacetyl formation with RKY2139 (WT, ▲) and RKY2315 (rho−, ○) (A) and sugar profiles and ethanol formation with RKY2139 (B) and RKY2315 (C) (the concentrations of maltose [●], glucose [▲], and ethanol [+] are indicated). Experiments were performed at least three times. The results of a representative experiment are shown.
The second band in the additional Ilv2 band was observed with the same mobility as targeting of matrix proteins (13). After CCCP addition, an mitochondrial membrane, which is essential for mitochondrial production observed in petite mutants. To prove that the pre-cytosol could be responsible for the enhanced diacetyl 

ings suggest that the accumulation of Ilv2 precursor protein in the cytosol is responsible for the enhanced diacetyl production observed in petite mutants. To prove that the precursor protein.

The Ilv2 precursor accumulates in the cytosol. These findings suggest that the accumulation of Ilv2 precursor protein in the cytosol could be responsible for the enhanced diacetyl production observed in petite mutants. To prove that the precursor is indeed localized to the cytosol and not to the mitochondrial matrix, a cell fractionation experiment was performed. Yeast cells were spheroplasted and gently lysed with a Dounce homogenizer. The cell extract was centrifuged at 100,000 × g and separated into a mitochondrial fraction containing P100 pellet and an S100 supernatant containing the soluble cytosolic proteins. To test for the integrity of mitochondria, the distribution of the mitochondrial matrix protein aconitase (Aco1) was examined (Fig. 2B). The protein was almost exclusively found in the pellet fraction. However, we consistently detected a small amount of mature Aco1 in the supernatant fraction (ca. 5% of the total protein). Evidence has been presented that a small fraction of Aco1 is localized to the cytosol (15). Therefore, it is difficult to decide whether this small amount of Aco1 in the supernatant results from the breakage of mitochondria or whether it represents a genuinely cytosolic Aco1 pool. The fractionation pattern of mature Ilv2 was very similar to the fractionation pattern of Aco1. The Ilv2 precursor, however, displayed a different fractionation behavior. The majority of the Ilv2 precursor was detected in the supernatant fraction (ca. 60%). Since the fraction of the Ilv2 precursor in the supernatant is much higher compared to mature Ilv2 or Aco1, we conclude that the Ilv2 precursor is to a large part localized to the cytosol.

If cytosolic Ilv2 is responsible for the high diacetyl levels in petite mutants, then expression of an import-incompetent Ilv2 variant should also lead to elevated diacetyl levels. To test this prediction, fermentations were performed with a diploid (rho−) yeast strain carrying one copy of the wild-type Ilv2 gene and one copy of a truncated version missing the first 55 codons. The truncated protein Ilv2Δ55 expressed from the mutant gene is missing the mitochondrial presequence and should not be imported into mitochondria. To prove that the Ilv2Δ55 protein is indeed localized to the cytosol, a cell fractionation experiment was performed (Fig. 2B). In contrast to the diploid wild-type strain, where only a small fraction of mature Ilv2 could be detected in the supernatant (7% of total protein), more than half of the processed/truncated Ilv2 protein (ca. 60% of total protein) was now found in the supernatant fraction. Thus, the fractionation experiment demonstrates that the truncated Ilv2Δ55 protein is not imported into mitochondria. (Since the Ilv2 precursor is presumably cleaved by MPP between amino acids 55 and 56 in the mitochondrial matrix, the processed and the truncated forms cannot be distinguished based on their gel mobility.)

With the ILV2/ILV2-Δ55 strain, diacetyl levels comparable to a rho− strain were detected (Fig. 3). This experiment, therefore, supports the notion that cytosolic localization of Ilv2 is responsible for the high diacetyl levels produced by petite mutants.

Reduced diacetyl formation by ATP3-5 expression. Since cytosolic accumulation of the Ilv2 precursor appears to be responsible for enhanced diacetyl production of petite yeasts, we were looking for a way to improve the efficiency of mitochondrial import of the precursor to reduce diacetyl production. The main reason for the reduced import efficiency of petite yeasts seems to be a lowered membrane potential (Δμ). In rho− yeasts, the membrane potential is maintained by exchange of matrix ADP3− for ATP3− by the ADP/ATP carrier. The ADP concentration in the mitochondrial matrix is raised...
by ATP hydrolysis by the F\(_1\)-ATPase, which stimulates the exchange activity of the ADP/ATP carrier. There is evidence that enhanced ATP hydrolysis by a mutation in the \(\gamma\)-subunit of the F\(_1\)-ATPase (\(ATP3-5\)) can boost \(\Delta\Psi\). This notion is based on the finding that the dominant \(ATP3-5\) mutation suppresses the petite-negative phenotype of a \(\Delta\text{yme1}\) mutant (21). Presumably, due to a negative effect on mitochondrial import, the \(\Delta\text{yme1}\) mutation is not compatible with the petite state.

To test the effect of \(ATP3-5\) on diacetyl formation of petite strains, a \(\rho^\circ\) strain was transformed with a plasmid expressing \(ATP3-5\) or as a control with a plasmid expressing wild-type \(ATP3\). Fermentations were carried out in SD/CAS medium with selection for the plasmid and in MEX medium without plasmid selection. In SD/CAS medium a clear reduction in diacetyl levels down to 40% of the wild-type control was observed with the \(ATP3-5\) transformant (Fig. 4A). In MEX medium, the effect was less pronounced but was nevertheless clearly detectable (reduction to 70% of control levels) (Fig. 4B). These experiments show that introduction of the \(ATP3-5\) allele into brewing yeast strains could be an interesting possibility to reduce diacetyl levels during beer fermentation.

**DISCUSSION**

We examined here the mechanisms that lead to increased diacetyl production by petite strains during beer fermentation. Our experiments suggest that diacetyl production is intimately linked to the mitochondrial import of acetohydroxyacid synthase, Ilv2. We could show that in petite mutants, which give rise to high diacetyl levels, the Ilv2 precursor protein accumulates in the cytosol. We propose that this cytosolic accumulation is the reason for the high diacetyl levels observed with petite yeasts. This notion is supported by the finding that expression of an Ilv2 variant with truncated signal sequence, which cannot be imported into mitochondria, leads to high diacetyl levels comparable to the levels observed with petite mutants. Also, it has been shown recently that cytosolic overexpression of the acetohydroxyacid reductoisomerase (Ilv5), the enzyme that functions immediately downstream of Ilv2 in the synthesis of branched-chain amino acids, reduces diacetyl levels (14).

Why should cytosolic localization of Ilv2 lead to elevated diacetyl levels in beer fermentations? In the case of cytosolic production, it may be easier for the diacetyl precursor \(\alpha\)-acetolactate to reach the surrounding culture medium, since it only has to be transported across one membrane, the plasma membrane, while in the case of production in the mitochondrial matrix, it would have to cross three different membranes (mitochondrial inner and outer membrane and the plasma membrane). The transporter responsible for \(\alpha\)-acetolactate membrane transport is not known. Maybe, there is no such transporter in the mitochondrial membranes. Another explanation could be that in the mitochondrial matrix the Ilv2 product \(\alpha\)-acetolactate is quickly consumed by the next enzyme in the Ilv pathway, the acetohydroxyacid reductoisomerase Ilv5. In the cytosol, Ilv5 may not be present under normal conditions; thus, \(\alpha\)-acetolactate could accumulate without being consumed by Ilv5. Yet another possibility is that Ilv2 activity in the mitochondrial matrix is negatively regulated by the regulatory subunit Ilv6. Again, Ilv6 may not be present in the cytosol; thus, Ilv2 activity could be higher than in the mitochondrial matrix under our growth conditions (rich medium containing valine, leucine, and isoleucine).

Ilv2 therefore appears to be another example of an enzyme with a dual localization that may be able to function in two different cellular compartments. Dual distribution between cytosol and mitochondria has been demonstrated for yeast fumarase (Fum1) (19). A fraction of fumarase returns to the cytosol after being processed in mitochondria. Rapid folding into an import-incompetent conformation seems to be crucial for retrograde movement (18). A similar competition between
spontaneous folding to an import-incompetent conformation and mitochondrial import also leads to a dual localization of yeast adenylate kinase (Adk1/Aky2) (20). A dual localization may not always be easily discernible. In the case of mitochondrial aconitase (Aco1) only minute amounts were detected in the cytosol, and yet this activity appears to be physiologically relevant (15). Cytosolic accumulation of matrix-targeted proteins in petite mutants is not a general phenomenon but appears to be connected to specific features of the protein. In this context, it is interesting that a conserved subclass of preproteins known to reside in internal mitochondrial compartments (including Ilv2) was detected in the mitochondrial outer membrane fraction (22). This specific localization may also be linked to site-specific localization and transcription of messenger RNAs on the surfaces of mitochondria (7).

It appears that a lowered membrane potential across the inner mitochondrial membrane is the reason for the elevated diacetyl levels observed with petite yeast strains. We show here that boosting the membrane potential by expressing the diacetyl levels observed with petite yeast strains. We show here ger RNAs on the surfaces of mitochondria (7).

ACKNOWLEDGMENT

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REFERENCES