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ARTICLE

Perdeuteration and methyl-selective ^{1}H, ^{13}C-labeling by using a *Kluyveromyces lactis* expression system

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Abstract The production of stable isotope-labeled proteins is critical in structural analyses of large molecular weight proteins using NMR. Although prokaryotic expression systems using *Escherichia coli* have been widely used for this purpose, yeast strains have also been useful for the expression of functional eukaryotic proteins. Recently, we reported a cost-effective stable isotope-labeled protein expression using the hemiascomycete yeast *Kluyveromyces lactis* (*K. lactis*), which allow us to express exogenous proteins at costs comparable to prokaryotic expression systems. Here, we report the successful production of highly deuterated (>90 %) protein in the *K. lactis* system. We also examined the methyl-selective ^{1}H, ^{13}C-labeling of Ile, Leu, and Val residues using commonly used amino acid precursors. The efficiency of ^{1}H- ^{13}C-incorporation varied significantly based on the amino acid. Although a high level of ^{1}H-^{13}C-incorporation was observed for the Ile δ1 position, ^{1}H, ^{13}C-labeling rates of Val and Leu methyl groups were limited due to the mitochondrial localization of enzymes involved in amino acid biosynthesis and the lack of transporters for α-ketoisovalerate in the mitochondrial membrane. In line with this notion, the co-expression with branched-chain-amino-acid aminotransferase in the cytosol significantly improved the incorporation rates of amino acid precursors. Although it would be less cost-effective, addition of ^{13}C-labeled valine can circumvent problems associated with precursors and achieve high level ^{1}H, ^{13}C-labeling of Val and Leu. Taken together, the *K. lactis* system would be a good alternative for expressing large eukaryotic proteins that need deuteration and/or the methyl-selective ^{1}H, ^{13}C-labeling for the sensitive detection of NMR resonances.

Keywords Stable-isotope labeling · Large molecular weight protein · Perdeuteration · Methyl-selective ^{1}H, ^{13}C-labeling · *Kluyveromyces lactis* · Eukaryotic expression system

Introduction

Obtaining a significant quantity of stable isotope-labeled proteins containing ^{15}N, ^{13}C, and/or ^{2}H is essential for NMR analyses of proteins. Prokaryotic protein expression systems using *Escherichia coli* (*E. coli*) are most widely used for this purpose, but the expression system is often not suitable for expressing large eukaryotic proteins, including those related to human diseases. Eukaryotic expression systems using insect and mammalian cells have been utilized to address this problem (Gossert et al. 2011; Sastry

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et al. 2011), but stable isotope labeling in these systems remains expensive. Yeast expression system is an attractive alternative for exogenous protein expression in eukaryotes (Morgan et al. 2000; Sugiki et al. 2008). Most posttranslational modifications in mammals are conserved in yeast and the stable isotope labeling is achieved with a simple set of nutrients. Recently, we proposed a yeast expression system using the hemiascomycete yeast *Kluyveromyces lactis* (*K. lactis*), which efficiently secretes heterologous proteins into culture medium (Sugiki et al. 2008). The *K. lactis* expression system uses *LAC4* promoters, which makes yeast grow and produce the protein of interest in the presence of glucose. This feature reduces the cost of stable isotope-labeled nutrients to levels almost comparable to those of prokaryotic systems (Sugiki et al. 2008).

Deuteration of protein is critical for NMR analyses of large molecular weight proteins. For example, the site-specific assignments using a standard set of 3D-techniques usually requires deuteration if the molecular mass of the targeting system exceeds 30 kDa. High-level deuteration of proteins is also useful to extract information about the intermolecular distance of large protein complexes (Gross et al. 2003; Takahashi et al. 2000). In addition, the selective protonation of methyl groups under high-deuteration background enabled us to obtain structural and dynamical information even from MDa protein systems (Tugarinov et al. 2007). Although the *K. lactis* system described above has been useful in expressing protonated uniformly-^{15}N, ^{13}C labeled proteins, its potential to express perdeuterated and methyl-selective ^{1}H, ^{13}C-labled proteins remains elusive.

Here, we show that the *K. lactis* expression system is useful for high-level deuteration (~95 %) and methyl-selective ^{1}H, ^{13}C-labeling using α-ketoacid precursors. Although the methyl-selective labeling of Leu and Val is not straightforward due to the subcellular localization of enzymes involved in amino acid synthesis and the low-level uptake of the precursor into mitochondria, we found that the overexpression of branched-chain-amino-acid aminotransferase (BCAT) can compensate the problem. In addition, high levels of ^{1}H, ^{13}C-labeling of Leu and Val can be achieved by supplementing Val into culture media. The successful deuteration and methyl-selective ^{1}H, ^{13}C-labeling achieved using the *K. lactis* expression system indicates that this system is a good alternative for structural and dynamical studies of large eukaryotic proteins and their complexes.

Materials and methods

Expression and purification of MBP in *K. Lactis*

A 42-kDa maltose binding protein (MBP), which has been extensively analyzed in *E. coli* for perdeuteration and methyl-selective protonation proteins was used in this study (Gardner et al. 1998). *K. lactis* cells harboring the MBP gene were prepared as previously described (Sugiki et al. 2008). The MBP protein was secreted in culture media and purified as previously described (Sugiki et al. 2008).

For the deuteration of MBP, the *K. lactis* cells harboring the MBP gene were first grown in 10 ml of H_2O YPD medium containing 1 % Yeast Extract, 2 % Bacto Peptone and 2 % glucose at 30 °C for 3 days (Primary culture). The yeast cells were then transferred to the 100 ml of D_2O YNB medium without ammonium sulfate and amino acids (Becton–Dickinson) supplemented with 2 g/l ammonium chloride and 10 g/l [U-D_7] glucose as nitrogen and carbon sources, respectively (Secondary culture). The cells continued to grow for 3 days with a constant air supply (500 ml/min) in an Erlenmeyer flask to adapt to D_2O. The incubation temperature was set to 30 °C and the flask was shaken at 250 rpm. D_2O adapted cells were then transferred to 400 ml of D_2O YNB minimal media containing 2 g/l of ammonium chloride and 6 g/l of [U-D_7] glucose as the nitrogen and carbon sources, respectively. The culture was incubated in a bioreactor vessel with a constant air supply (1.5 l/min) at 30 °C for 3 days with vigorous vortex (800 rpm). During the culture period, 600 ml of fresh minimal media containing 4.3 g/l [U-D_7] glucose was added at a constant flow rate of 8.3 ml/h with a peristaltic pump. The final concentration of [U-D_7] glucose was 5.0 g/l (Fig. 1a, right). For "non-adapted" cells, the primary culture grown in 10 ml of H_2O YPD medium was directly transferred to final D_2O YNB minimal media (Fig. 1a, left). For methyl-selective ^{1}H, ^{13}C-labeling, 100 mg/l of amino acid precursors ([methyl-^{13}C, 3,3-D_2] α-ketobutyrate and [3-methyl-^{13}C, 3,4,4,4-D_4] α-ketoisovalerate) were supplemented to the both basal and feeding media.

Co-transfection of branched-chain-amino-acid aminotransferase (BCAT) and MBP genes

The co-transfections of BCAT and MBP genes were performed according to the manufacturer's protocol. For expression of bacterial and cytosolic BCATs, the gene coding *E. coli* IlvE and *S. cerevisiae* BAT2 were used, respectively. The presence of both genes in transfected *K. lactis* cells were confirmed by quantitative PCR. *K. lactis* genomic DNA containing exogenous genes was prepared using the Bust'n Grab method (Harju et al. 2004). The quantitative PCR reactions were performed using SYBR Premix Ex Taq II according to the manufacturer's protocol. The following primer sequences were used in quantitative PCR: 5′-tggattaacggcgataaagg-3′ and 5′-agataatgtcagggccatcg-3′ for MBP; 5′-gtacctggcggatgaagtgt-3′ and 5′-gccccatttatcttcggttt-3′ for IlvE; and 5′-aaaacgggcaagaaggaact-3′ and 5′-caatcgcagcagtaccagaa-3′ for Bat2.

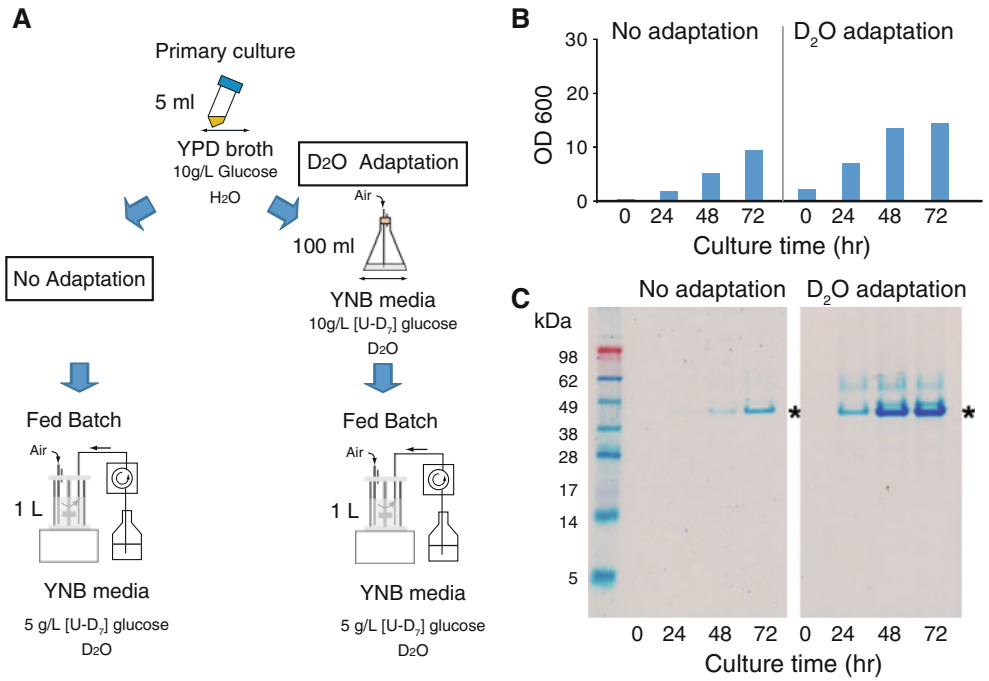

Fig. 1 Expression of the deuterated MBP in *K. lactis*. **a** The experimental scheme for deuterated protein expression. **b**, **c** Comparison of **b** the growth and **c** MBP expression of D_2O-adapted and non-adapted *K. lactis* cells using "fed-batch" expression procedures

NMR experiments

All NMR experiments were conducted using samples containing 10 mM deuterated HEPES–NaOH (pH 7.4) with 1 mM β-cyclodextrin. The D_2O ratios of samples were set to 10 or 99.6 % depending on the type of experiment. All NMR experiments were performed on a Bruker Avance 600 MHz spectrometer equipped with a cryogenic TXI probe. 2D ^{1}H-^{13}C-HMQC experiments were recorded with a spectral width of 4,527 Hz for carbon (centered at 15 ppm) and 8,389 Hz for proton (centered at 4.7 ppm). 512 and 300 complex data points were recorded for direct and indirect dimensions, respectively. The repetition delay was set to 1.2 s. All experiments were performed at 298 K, and NMR data were processed using TopSpin 2.1 and analyzed in SPARKY.

Results and discussion

Production of deuterated protein by *K. lactis*

To determine whether highly deuterated protein could be produced using the *K. lactis* expression system, the expression of MBP in D_2O YNB minimal media containing deuterated glucose was examined. Previously, we reported that the ''Fed-batch'' strategy, in which nutrients are supplied into the culture media in a controlled manner, minimizes the amount of glucose in cell culture media without sacrificing expression levels (Sugiki et al. 2008). The same strategy was used here for the efficient expression of deuterated proteins. Cells pre-adapted to D_2O containing rich media have also been reported to grow faster and express more protein than unadapted cells in the *Pichia pastoris* expression system (Morgan et al. 2000). Therefore, we compared cell growth and the expression of MBP in *K. lactis* with (Fig. 1a, right) and without (Fig. 1a, left) preadaptation in a small amount of D_2O YNB medium.

As described in detail in the Materials and Methods section, primary cultures of *K. lactis* harboring a MBP gene were transferred to D_2O YNB medium supplemented with 10 g/l [U-D_7] glucose as the carbon source. These cultures continued to grow for 3 days at 30 °C to adapt to D_2O. D_2O-adapted cells were then transferred to 400 ml of minimal media containing 6 g/l [U-D_7] glucose and 600 ml of fresh minimal media containing 4.3 g/l [U-D_7] glucose was added at a constant flow rate during a 3-day inoculation period (Fig. 1a, right). For "non-adapted" cells, primary cultures grown in 10 ml of H_2O YPD medium were directly transferred to the final D_2O YNB minimal media and cultured using the ''Fed-batch'' method (Fig. 1a, left).

Cell growth and expression of perdeuterated MBP are summarized in Figs. 1b, c, respectively. As expected, the D_2O-adapted cells grew faster compared to non-adapted cells. In addition, the protein yield from the adapted cells improved by approximately three times compared to that of non-adapted cells. Under these culture conditions, the

expression of perdeuterated MBP from 1 L culture was 3.6 mg. As expected, the yield was less compared to fully protonated cultures (10 mg/ml) (Sugiki et al. 2008), however, is enough to make a 300 μM sample from single batch of culture (1 L).

The 1D ^{1}H NMR spectrum of perdeuterated MBP obtained using this procedure is shown in Fig. 2a. Compared to the unlabeled (protonated) counterpart in Fig. 2b, a high level deuteration of non-exchangeable protons including methyl, methylene, as well as aromatic positions is obvious from the minimal amount of signals in the aliphatic as well as aromatic regions (Fig. 2a). The deuteration rate judged from the intensity of aliphatic proton resonance was 95 %, which was consistent with the average deuteration rate estimated from a mass spectroscopic analysis using MALDI-TOF (92 %). Previously, about 80 % overall deuteration rate, which is significantly lower compared to our study using *K. lactis*, was reported for a protein expressed using *P. pastoris* in 95 % D_2O media containing deuterated methanol (Morgan et al. 2000). The NMR analysis of the deuterated protein expressed in *P. pastoris* indicated that significant numbers of side chain protons were left protonated. However, the deuteration rate of the side chain protons is significantly higher in our study indicating more uniform and high-level deuteration is achieved with the *K. lactis* expression system.

Methyl-selective ^{1}H, ^{13}C-labeling of the Ile, Leu, and Val residues using *K. lactis*

The accomplishment of high-level deuteration motivated us to explore selective ^{1}H, ^{13}C-labeling of Ile, Leu, and Val methyl groups under deuterated backgrounds. Although amino acid synthesis pathways are common in prokaryotic and eukaryotic cells, several essential enzymes are exclusively located inside mitochondria in eukaryotic cells (Solà et al. 2007, 2004). Furthermore, the transportation of solutes across the mitochondrial membrane is strictly regulated in eukaryotes. These factors can potentially limit the use of the amino acid precursors, especially for those metabolized by a mitochondria-localized enzyme.

Figure 3 shows the amino acid synthesis pathway leading to Ile, Leu and Val. Acetohydroxy acid synthase, isomero-reductase, dihydroxy acid dehydratase, and BCAT are involved in the biosynthesis of branched-chain amino acids. In *Saccharomyces cerevisiae*, all of these enzymes, with the exception of BCAT, are localized in the mitochondria (Ryan and Kohlhaw 1974). *S. cerevisiae* has a paralogous pair of BCAT enzymes BAT1 and BAT2, which are localized in the mitochondria and the cytoplasm, respectively. Although, there is limited information about the subcellular localization of these enzymes in *K. lactis*, a recent paper described that there is only one BCAT in the strain localized in the mitochondria (Colón et al. 2011). The localization is due to the lineage, which gave rise to *K. lactis*, diverging before a whole genome duplication event. As a result, the *K. lactis* genome does not contain the duplication blocks present in *S. cerevisiae* (Kellis et al. 2004). Therefore, all the *K. lactis* enzymes that are used to synthesize Ile and Val are most likely localized in the mitochondria. Leu is derived from the Val biosynthetic pathway as the amino acids share α-ketoisovalerate as a common precursor. The Leu biosynthetic pathway needs several specific enzymes and is thus more complicated. Among the Leu-specific enzymes, only α-isopropylmalate (α-IPM) synthase is shown to be localized in the mitochondria, whereas IPM isomerase and β-IPM dehydrogenase are considered to be cytoplasmic (Ryan et al. 1973). The final enzyme, BCAT, is shared among Ile, Leu, and Val biosynthetic pathways.

To determine whether we can selectively label the methyl groups of amino acids, two commonly used amino acid precursors, α-ketobutyrate (for Ile labeling) and α-ketoisovalerate (for Leu and Val labeling) were investigated. Figure 4b shows the ^{1}H-^{13}C HMQC spectrum of methyl-selective ^{1}H, ^{13}C-labeled MBP expressed in *K. lactis* using the "Fed-batch" deuteration procedure. Compared to the *E. coli*-expressed counterpart shown in Fig. 4a, Ile δ1 was extensively labeled with amino acid precursors, whereas the incorporation of α-ketoisovalerate to Leu δ and Val γ seemed to be quite limited in the *K. lactis* expression system. The ^{1}H, ^{13}C-labeling rates of the Ile δ1,

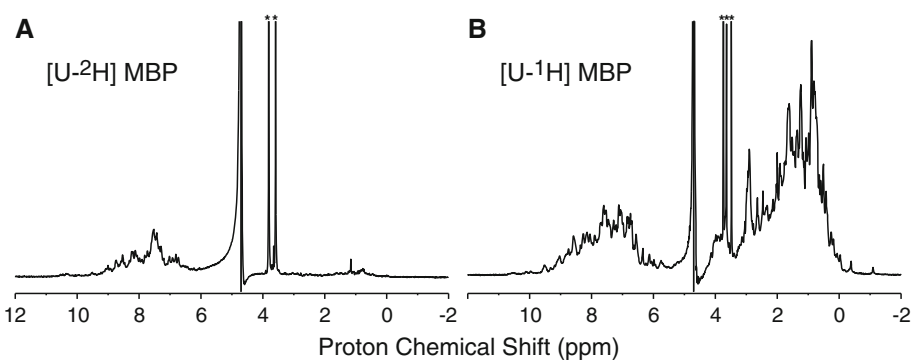

Fig. 2 1D ^{1}H NMR spectrum of **a** deuterated and **b** unlabeled MBP from the *K. lactis* expression system. MBP (45 μM) complexed with 1 mM β-cyclodextrin was dissolved in 10 mM deuterated HEPES–NaOH (pH 7.4). Resonances from β-cyclodextrin were indicated with *asterisks*. Both spectra were recorded under the same conditions

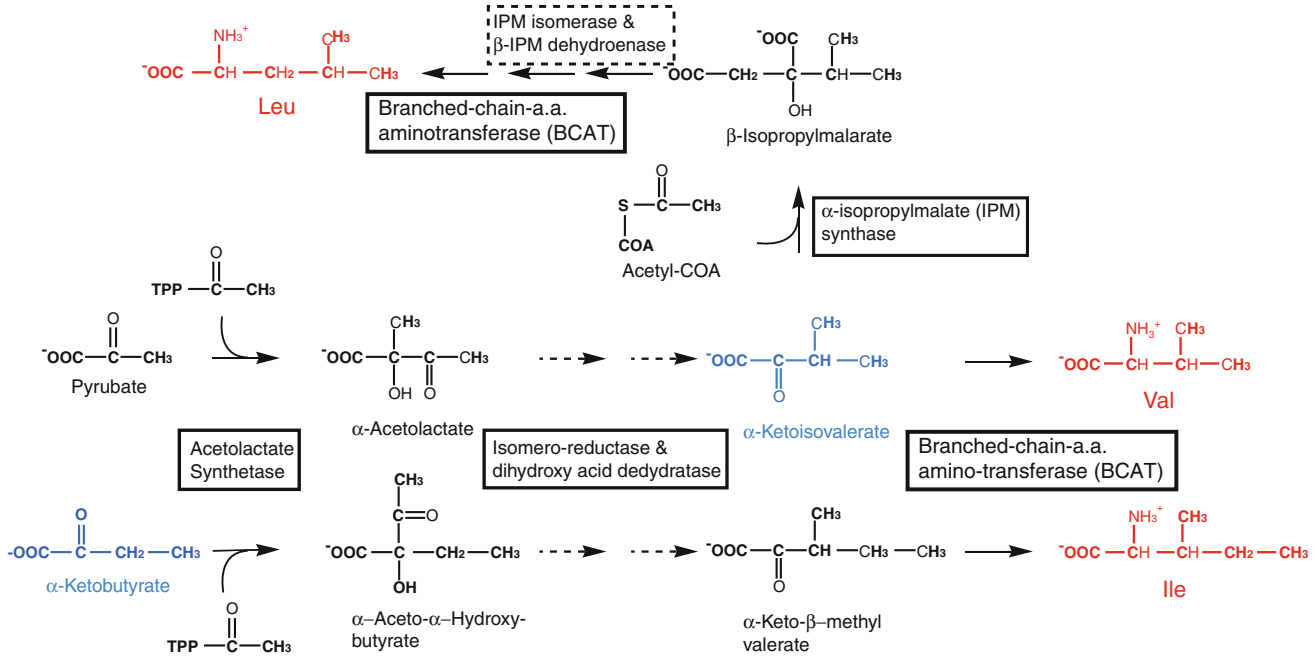

Fig. 3 Amino acid synthetic pathways leading to isoleucine, valine, and leucine. The enzymes that catalyze each step of these pathways are indicated in *boxes*. The *solid* and *broken squares* represent the putative localization of each enzyme in yeast to the mitochondria and cytosol, respectively. Structures in colored in *blue* indicate the amino acid precursors α-ketobutyrate and α-ketoisovalerate, which are commonly used in prokaryotic systems. Branched-chain-a.a.-amino-transferese shown in the figure corresponds to IlvE in bacteria and BCATs in yeast

Leu δ, and Val γ positions were calculated based on the intensity of each resonance in the HMQC spectra relative to *E. coli*-expressed ILV-labeled MBP and summarized in Table I. There are no CH_2D and CHD_2 isotopomers observed in the labeling scheme. Increasing the amount of precursor in the media or using another precursor for Leu and Val, α-acetolactate, did not significantly improve the labeling rates of Leu δ and Val γ positions (data not shown).

As discussed above, all *K. lactis* enzymes involved in Ile and Val biosynthesis are shared and localized in the mitochondria. Therefore, we hypothesized that the low incorporation of α-ketoisovalerate to produce Val and Leu is a result of inefficient translocation of the precursor into the mitochondria. Indeed, when 100 mg/L of [U-^{13}C] Val was supplemented to D_2O YNB medium, Val was successfully converted to Leu and both ^{13}C-labeled amino acids were clearly incorporated into the protein (Fig. S1). The ^{1}H, ^{13}C incorporation rates for Leu and Val methyl positions were estimated to be approximately 85 and 95 %, respectively, compared to those of proteins expressed using the *E. coli* system with the same amount of [U-^{13}C] Val (Table 1).

The successful conversion of Val to Leu in *K. lactis* led us to overexpress BCAT in cytosol. Because α-ketoisovalerate is directly converted to Val by BCAT, we hypothesized that the overexpression of this enzyme in cytosol should increase the cellular concentration of Val, which in turn is utilized to produce Leu. To test our hypothesis, a plasmid harboring the bacterial BCAT, IlvE, as well as, the cytosolic BCAT from *S. cerevisiae*, BCAT2, were co-transfected into cells with MBP plasmid. The successful integration of the BCAT and the MBP gene into the yeast genome was confirmed by quantitative PCR. Clones with the highest copy number of BCAT and sufficient expression levels of MBP were selected among 24 isolated clones for each enzyme.

Figure 4b, c show ^{1}H-^{13}C HMQC spectra of methyl-selective ^{1}H, ^{13}C labeled MBPs expressed in *K. lactis* co-expressing IlvE and BCAT2, respectively. The labeling rates of Ile δ1, Leu δ, and Val γ under BCAT overexpression are indicated in Table 1. The BCAT overexpression improved the incorporation rates of amino acid precursors. The labeling rates of Ile were greater than 80 % in both cases, whereas Leu and Val labeling rates were approximately 10 and 15 %, respectively. Improved incorporation of Ile was somewhat unexpected as this indicates that BCAT overexpression improved the efficiency of whole branched amino acid synthetic pathways. As expected, improved labeling rates of Leu and Val were observed. The lower incorporation of Leu compared to that of Val may reflect its complicated synthetic pathway. Thus,

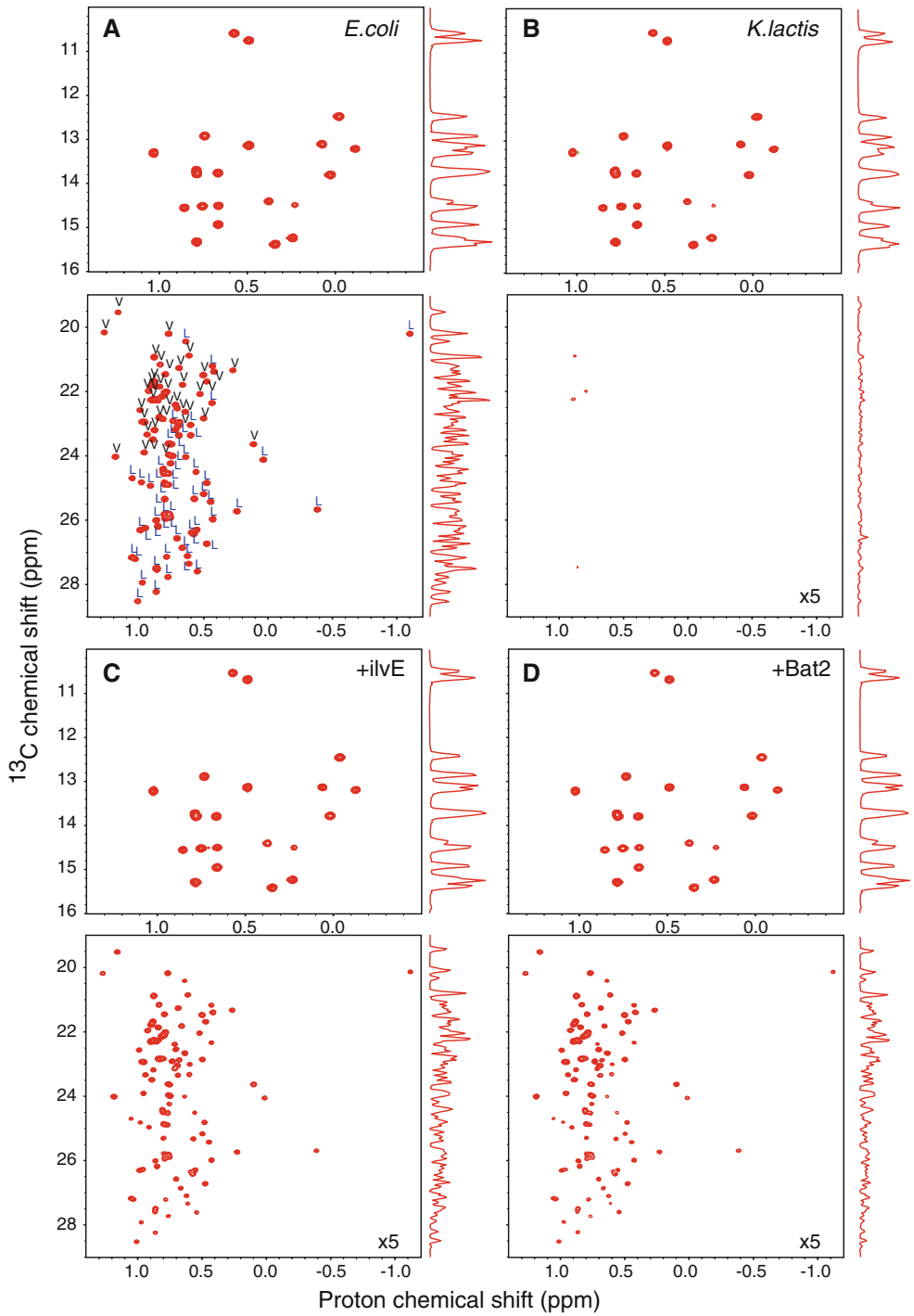

Fig. 4 Comparison of HMQC spectra of ILV-methyl ^{1}H, ^{13}C-labeled perdeuterated MBP. **a** Reference HMQC spectra of the ILV-methyl ^{1}H, ^{13}C-labeled perdeuterated MBP, which were expressed in *E. coli*. **b–d** ILV-methyl ^{1}H, ^{13}C-labeled perdeuterated MBP produced using the *K. lactis* system. **c**, **d** IlvE and Bat2 overexpression, respectively. The *upper* spectra in each *panel* correspond to Ile δ1 signals, while the *lower* spectra show Leu and Val methyl signals. In the *lower* spectra, the originating amino acid residue types are indicated. Spectra were normalized by concentration and the ^{13}C-projection for each spectrum is shown on the *right*. For spectra recorded for MBP produced using the *K. lactis* system, Leu and Val regions were shown with a 5-fold lower base line levels and the 13C-projection was enlarged by 5-fold. 2D ^{1}H-^{13}C-HMQC experiments were recorded with a spectral width of 4,527 Hz for carbon (centered at 15 ppm) and 8,389 Hz for proton (centered at 4.7 ppm). 512 and 300 complex data points were recorded for direct and indirect dimensions, respectively. The repetition delay was set to 1.2 s. Four scans were used, and the experiment was completed within 73 min

Table 1 Summary of ^{1}H-^{13}C-incorporation rates using various expression conditions

	Ile	Leu	Val
Labeled with ILV precursors[a]			
WT *K. Lactis* cell	67 ± 6 %	2.2 ± 0.3 %	2.4 ± 0.4 %
IlvE overexpression	88 ± 7 %	10 ± 2 %	15 ± 3 %
Bat2 overexpression	78 ± 6 %	8 ± 1 %	14 ± 3 %
Labeled with Val[b]			
WT *K. Lactis* cell	N.D.	87 ± 11 %	94 ± 9 %

[a] The ^{1}H-^{13}C-incorporation rates in Ile δ1, Leu δ, and Val γ positions were calculated using the intensity of each resonance in the HMQC spectra of the *K. lactis*-expressed ILV-methyl ^{1}H, ^{13}C-labeled perdeuterated MBP relative to its *E. coli*-expressed counterpart. [b]The ^{1}H-^{13}C-incorporation rates in Leu δ, and Val γ positions were calculated using the intensity of each resonance in the HMQC spectra of the *K. lactis*-expressed LV-^{1}H, ^{13}C-labeled perdeuterated MBP relative to its *E. coli*-expressed counterpart. The average and SD of ^{1}H-^{13}C-incorporation rates were calculated for non-overlapping signals

Val can be successfully produced in yeast cytoplasm by BCAT enzymes. The absolute labeling rate for Val was not as high as that of Ile, which may due to the dilution of produced Val in culture media.

Conclusion

In this work, we found that the *K. lactis* expression system can be used for high-level (>90 %) and uniform deuteration of proteins, which are critical for NMR analysis of large molecular weight systems. The uniform and high-level deuteration observed in this study is suitable for fastidious but effective experiments, such as forbidden coherence transfer, which utilizes cross-correlated relaxation effects in isolated methyl signals (Tugarinov et al. 2003, 2007). This expression system can also be used for cross-saturation experiments, which facilitates the precise determination of binding interfaces (Matsumoto et al. 2010; Shimada et al. 2009).

We further investigated whether the selective labeling of Ile (δ1), Leu, and Val methyl groups in highly deuterated backgrounds could be achieved using this expression system. Although the efficiency of ^{1}H-^{13}C-incorporation varied significantly based on the amino acids and precursor used, two commonly used amino acid precursors, α-ketobutyrate (for Ile labeling) and α-ketoisovalerate (for Leu and Val labeling) were successfully incorporated into the protein. Leu and Val showed lower levels of incorporation compared to that of Ile, demonstrating the lack of α-ketoisovalerate specific transporters in the mitochondrial membrane as well as the subcellular localization of enzymes involved in amino acid biosynthesis. Incorporation into Leu was consistently lower, which was expected considering its complex biosynthetic pathway. Overexpression of both a bacterial and cytosolic BCAT significantly improved the incorporation rates of amino acid precursors. The labeling rate of Ile was close to 100 % and the Leu and Val labeling rates were approximately 10 and 15 %, respectively. Although the labeling rates of Leu and Val were not high, preferable relaxation properties of the methyl probes allow us to detect resonances with sufficient signal-to-noise ratios in approximately 1 h with 40-μM sample. If high-level ^{1}H, ^{13}C-labeling of Val and Leu is needed, one can use [2,3-D_2, U-^{13}C] Val, although this way would be less cost-effective. In summary, *K. lactis* is suitable for producing highly deuterated and selective methyl-labeled proteins, providing a new tool for structural analyses of large and eukaryotic proteins that are important but hard to tackle using current prokaryote based-expression systems.

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