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Notes & Tips

Isotopic labeling of proteins by utilizing photosynthetic bacteria

Hiroaki Suzuki [a,b], Yuichiro Shimada [b], Masayuki Kobayashi [c], Masato Kudo [b], Tsunenori Nozawa [d], Zheng-Yu Wang [a,b,*]

[a] *Faculty of Science, Ibaraki University, Mito 310-8512, Japan*
[b] *Department of Biomolecular Engineering, Tohoku University, Sendai 980-8579, Japan*
[c] *Ariake National College of Technology, Omuta, Fukuoka 836-8585, Japan*
[d] *National Institution for Academic Degree and University Evaluation, 1-29-1 Gakuen-nishimachi Kodaira-shi, Tokyo 187-8587, Japan*

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Determination of the three-dimensional solution structures of proteins is essential to the understanding of protein functions and interactions which could lead to new pharmaceutical developments. Novel high-resolution nuclear magnetic resonance (NMR)[1] techniques have emerged and provided powerful tools to analyze structures of proteins of up to ~900 kDa molecular weight [1]. These state-of-the-art experiments require the use of recombinant proteins labeled with stable isotopes, most notably ^{15}N, ^{13}C, and ^{2}H. The most widely used method to obtain isotope-enriched proteins has been through the expression of proteins from bacterial cells grown in minimal media containing ^{13}C-enriched glucose and ^{15}N-enriched salts. Although this method has proven successful in the determination of many protein solution structures by NMR, there are limitations associated with using minimal media for protein expression. Growth rates and protein expression can be increased with the use of "rich media" products that contain more complex substrates rather than simple sugars and nitrogen salts [2]. The rich media that contain mixtures of amino acids, peptides, nuclear acids, and complex carbohydrates can decrease doubling times and increase total protein expression. As a result, these media improve efficiency of the total labeling process and increase the yields of the proteins of interest. Several cell growth media products derived from algae have been commercially available. However, the high cost is still one of the limitations, and researchers need to conduct their own tests to determine the optimal conditions prior to purchase because favorable results may not always be obtained. In this study, we present a simple and efficient method of utilizing photosynthetic bacteria to produce isotopically labeled cultures for *Escherichia coli* expression systems. Photosynthetic organisms have been widely used to produce various biochemical compounds of high commercial value [3–6]. Photosynthetic bacteria can grow in closed batch cultures supplied with simple carbon and nitrogen sources for the synthesis of their biomass and can accumulate a large proportion (>60% w/w in dry cells) of proteins, sugars, and lipids [7]. Three photosynthetic bacteria species have been examined in this work and their applicability for expressing three photosynthetic membrane proteins in *E. coli* has been demonstrated using the isotope-labeled media.

^{15}N-labeled cultures were obtained using photosynthetic purple nonsulfur bacterium *Rhodospirillum* (*R.*) *rubrum* (ATCC No. 11170) cells grown in a medium (pH 6.8) containing 6.0 g/L DL-malic acid as the sole carbon source and 0.42–2.5 g/L NH_4Cl as the nitrogen source [8]. To obtain ^{13}C and ^{15}N doubly labeled cultures, the DL-malic acid in the above medium was replaced by $^{13}CH_3{}^{13}COONa$ ($^{13}C > 99\%$) at a concentration of 0.8 g/L. The cells were grown under continuous illumination with a 60-W incandescent lamp at 10 cm distance at 30 °C for 2 and 4 weeks for the ^{15}N and $^{15}N/^{13}C$ media, respectively [9]. Another two sulfur bacteria species were used for preparing ^{13}C and ^{15}N doubly labeled cultures.

* Corresponding author. Fax: +81 29 228 8352.
E-mail address: wang@mx.ibaraki.ac.jp (Z.-Y. Wang).

[1] *Abbreviations used: Ach*, *Allochromatium*; *Chl*, *Chlorobium*; HPLC, high-performance liquid chromatography; HSQC, heteronuclear single-quantum correlation; LH, light-harvesting; NMR, nuclear magnetic resonance; *R*, *Rhodospirillum*.

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Purple bacterium *Allochromatium* (*Ach.*) *vinosum* (ATCC No. 35206) was cultured anaerobically in a medium containing 1.0–2.0 g/L $NaHCO_3$ as the sole carbon source and 0.5–1.0 g/L NH_4Cl as the nitrogen source in addition to 0.1% Na_2S, 0.2% $Na_2S_2O_3$, and other inorganic salts [10]. Thermophilic green bacterium *Chlorobium* (*Chl.*) *tepidum* (ATCC No. 49652) was grown anaerobically at 48 °C in a medium consisting of 1–2 g/L $NaHCO_3$ and 0.3–0.5 g/L acetic acid as the carbon sources and 0.4 g/L NH_4Cl as the nitrogen source [11]. The cells were harvested by centrifugation (10,000*g*) at 4 °C for 10 min, followed by hydrolysis with 6 N HCl (20 mL/g, wet cells) at 110 °C for 24 h. The acidic solution containing the hydrolysates was first evaporated and then evacuated by vacuum pump to remove the residual HCl. The pellet was suspended with distilled water, followed by neutralization with NaOH to pH 7.0 and centrifugation to remove insoluble materials.

The hydrolysates were diluted with M9 medium in which the carbon and nitrogen sources were eliminated. The final culture contained different amounts of hydrolysates and various mineral salts as follows: 15 g/L $Na_2HPO_4(12H_2O)$, 3 g/L KH_2PO_4(anhydrous), 0.5 g/L NaCl, 0.24 g/L $MgSO_4$, 14.7 mg/L $CaCl_2$, 10 mg/L vitamin B_1, 10 mg/L thiamine. Three target proteins were used to examine the applicability of the cultures prepared from the hydrolysates. The light-harvesting integral membrane apoproteins, LH1α and LH1β, of *R. rubrum* and a photosynthetic membrane protein, PufX, of *Rhodobacter sphaeroides* were cloned as C-terminal His-tag fusions in pET-20b(+) (Novagen, WI, USA) and expressed in *E. coli* strain B21(DE3)pLysS by the same method described previously [12].

Table 1 shows a comparison of the amounts of carbon and nitrogen compounds required for yielding 10 g of wet cells of photosynthetic bacteria. All three bacteria species can grow in the media with ammonium chloride as the sole nitrogen source and simple compounds as carbon sources. Therefore, these bacteria can be used for ^{15}N or $^{15}N/^{13}C$ labeling of the proteins. The results were obtained by adjusting the amounts of carbon and nitrogen sources in various compositions and represent optimal conditions for the most efficient growth of the bacteria. *R. rubrum* grown in the medium supplied with malic acid as the carbon source is considered most economical for producing the uniformly ^{15}N-labeled culture because the least amount of ammonium chloride is required, while for the purpose of $^{15}N/^{13}C$ labeling three options with different requirements for the carbon sources are offered. *R. rubrum* grown in the medium with sodium acetate can be used not only for preparing uniformly ^{13}C-labeled culture but also for selectively labeling proteins with different labeling patterns of carbon atom if using either $^{13}CH_3COONa$ or $CH_3{}^{13}COONa$ as the carbon source. For example, the ε1 carbon of the imidazole side chain of histidine residue was found to be preferentially labeled by using $^{13}CH_3COONa$ (data not shown). On the other hand, *A. vinosum* utilizes sodium bicarbonate as the sole carbon source and is suitable for producing the uniformly $^{15}N/^{13}C$-labeled culture. The *C. tepidum* requires both sodium bicarbonate and sodium acetate as the carbon sources, and therefore several combinations of ^{13}C-labeling pattern can be prepared in addition to the uniform labeling as demonstrated previously [13].

Table 1
Comparison of the amounts (g) of carbon and nitrogen sources and culture volumes (L) required for yielding 10 g of wet cells of photosynthetic bacteria

	R. rubrum (1)[a]	*R. rubrum* (2)[a]	*A. vinosum*	*C. tepidum*
$^{15}NH_4Cl$	0.8	3.2	3.2	2.7
$NaH^{13}CO_3$	—	—	9.4	6.7
$^{13}CH_3{}^{13}COONa$	—	3.2	—	2.0
DL-Malic acid	6.0	—	—	—
Culture volume	1.2	4.8	4.8	4.0

[a] *R. rubrum* can utilize either acetic acid or malic acid as the sole carbon source.

The hydrolysates prepared from various photosynthetic bacteria were examined by the *E. coli* expression system. There was no apparent difference in the performance among these hydrolysates. A typical result of *E. coli* growth is shown in Fig. 1A using the hydrolysates prepared from *R. rubrum* cells. For comparison, growth curves of *E. coli* in M9 and LB media are also shown in the same figure under the same experimental conditions. The growth rate is significantly enhanced with respect to that in the M9 medium by addition of the hydrolysates and strongly depends on the concentration of the hydrolysates. Overall yield of protein expressed by the method of this study was estimated to be at least three times greater than that using M9 medium. The result can be explained on the basis of amino acid analysis. The ninhydrin assay revealed that the total amino acid concentration was about 1 mM in 1 L culture containing the hydrolysates prepared from 50 g of wet cells. This concentration is about the same as that assayed for the LB medium. Leucine, isoleucine, alanine, aspartic acid, glutamic acid, and valine were found most abundant in the hydrolysates. For the general purpose of NMR structural analysis with the *E. coli* expression system, induction is usually conducted when the cell density reaches to about $OD_{600} = 1$. This condition can be achieved by using the hydrolysates derived from 20 g of wet cells for preparing 1 L medium. The *E. coli* growth rate increased drastically when supplemented with glucose to the hydrolysate medium as shown in Fig. 1A, and this may be particularly useful for producing uniformly ^{15}N-labeled medium.

We have expressed three membrane proteins, LH1α, LH1β, and PufX, in *E. coli* using the hydrolysates derived from isotope-labeled photosynthetic bacterial cells. Fig. 1B shows the two-dimensional ^1H-^{15}N HSQC spectra for the LH1β protein. Backbone amide resonances were clearly observed for both proteins, indicat-

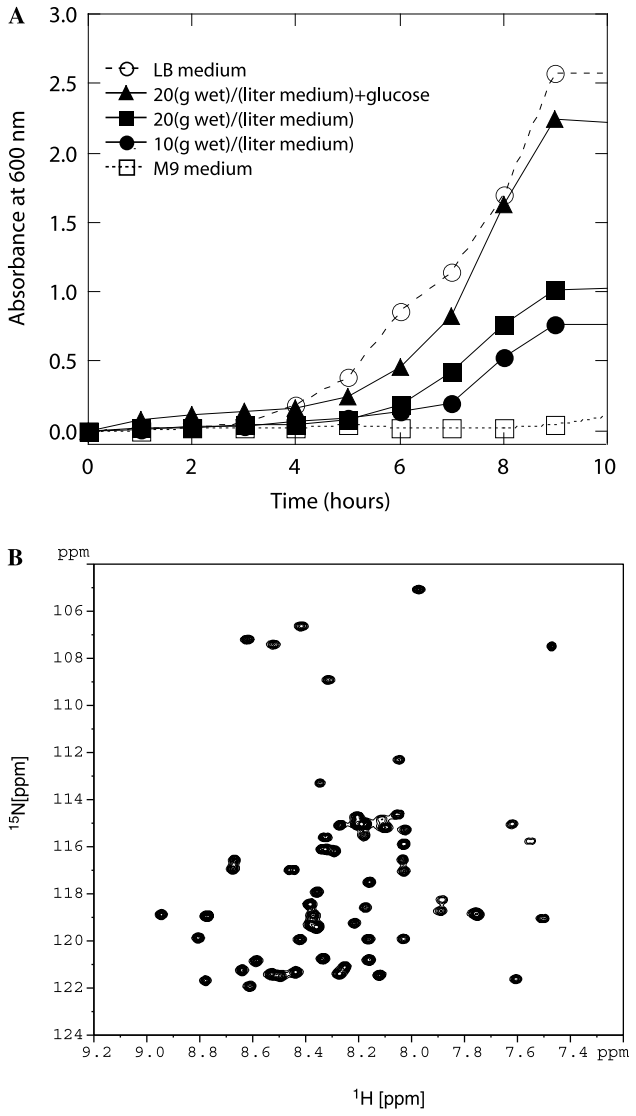

Fig. 1. (A) Comparison of the growth rates using LB, M9, and various cultures derived from photosynthetic bacterium *R. rubrum*. (B) Two-dimensional ^{1}H–^{15}N HSQC spectrum of *R. rubrum* LH1β membrane protein expressed in *E. coli* using the hydrolysates of ^{15}N-labeled *R. rubrum* cells.

ing that the isotope-labeling method developed in this study can be efficiently applied to the *E. coli* expression system. It is of interest to note that the NH chemical shift patterns of the expressed proteins are strikingly similar but not completely identical to those assigned for the corresponding native proteins [9]. We are continuing to investigate the structural difference.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2005.05.012.

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