Growth of wildtype and mutant E. coli strains in minimal media for optimal production of nucleic acids for preparing labeled nucleotides

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Abstract Since RNAs lie at the center of most cellular processes, there is a need for synthesizing large amounts of RNAs made from stable isotope-labeled nucleotides to advance the study of their structure and dynamics by nuclear magnetic resonance (NMR) spectroscopy. A particularly effective means of obtaining labeled nucleotides is to harvest these nucleotides from bacteria grown in defined minimal media supplemented with 15NH4Cl and various carbon sources. Given the high cost of carbon precursors required for labeling nucleic acids for NMR studies, it becomes important to evaluate the optimal growth for commonly used strains under standard minimal media conditions. Such information is lacking. In this study, we characterize the growth for Escherichia coli strains K12, K10zwf, and DL323 in three minimal media with isotopically labeled carbon sources of acetate, glycerol, and glycerol combined with formate. Of the three media, the LeMaster-Richards and the Studier media outperform the commonly used M9 media and both support optimal growth of E. coli for the production of nucleotides. However, the growth of all three E. coli strains in acetate is reduced almost twofold compared to growth in glycerol. Analysis of the metabolic pathway and previous gene array studies help to explain this differential growth in glycerol and acetate. These studies should benefit efforts to make selective 13C-15N isotopic-labeled nucleotides for synthesizing biologically important RNAs.

Keywords Escherichia coli · K12 · K10zwf · DL323 · BL21 · Minimal media · Glycerol · Acetate · Formate

Introduction

Various methods exist for labeling nucleic acids and proteins with stable isotopes for structural and dynamics studies (Dayie 2008; Hoogstraten and Johnson 2008; Lu et al. 2010). Escherichia coli is a commonly used bacterial host for protein production and for making RNA (Ponchon and Dardel 2007; Ponchon et al. 2009). E. coli can grow in chemically defined minimal media supplemented with 13N-labeled nitrogen and 13C-labeled carbon sources. The total cellular RNA obtained from such growth is enzymatically digested to nucleoside monophosphates (NMPs) and the NMPs are rephosphorylated to the nucleoside triphosphates (NTPs). These labeled NTPs then serve as the building blocks for making labeled RNA of any desired sequence using T7 RNA polymerase-based transcription (Milligan and Uhlenbeck 1989; Batey et al. 1992; Batey et al. 1995).

Previously, 13C or 13C/15N labeled nucleotides were produced by growing the bacteria Methylophilus methylotrophus (Batey et al. 1992) or Methylobacterium extorquens (Hines et al. 1994) on 13C-methanol as the most economical source of 13C-labeled carbon. At that time, the use of these bacteria that grow on methanol had the singular advantage of reduced cost over E. coli (Batey et al. 1995), but it is no longer advantageous for a number of reasons. First, isotopic scrambling occurs during biosynthesis and precludes the incorporation of site-specific isotopic labels using metha-
In addition to these cost considerations, a number of E. coli strains offer the potential for uniform and alternate site-specific $^{13}$C isotopic labeling. For example, the growth of wildtype E. coli strain K12 on sodium $^{13}$C-2-acetate ($^{13}$CH$_3$COONa) leads to >90% labeling efficiency at the C5 and C6 carbon positions in the pyrimidine rings and at the C2, C5, and C8 carbon positions within the purine rings (Hoffman and Holland 1995). Similarly, growth on $^{13}$C-1, 3-glycerol yields ~90% $^{13}$C isotopic enrichment at the C5 position of pyrimidines for mutant E. coli strain DL323 deficient in two tricarboxylic acid (TCA) cycle enzymes: succinate dehydrogenase (sdh) and malate dehydrogenase (mdh). Likewise, growth of DL323 on $^{13}$C-2-glycerol yields ~90% $^{13}$C isotopic enrichment at the C6 position of pyrimidines (Johnson et al. 2006). In contrast, growth on $^{13}$C-2-glycerol labels not only the C2' (~90%) and C4' (~90%) ribose positions, but also the C5 and C6 positions of pyrimidines mutually exclusively for another E. coli strain K10-1516 (hereafter referred to as K10zwf). K10zwf is deficient in the glucose-6-phosphate dehydrogenase enzyme. Unlike K12 and DL323, the growth of K10zwf on $^{13}$C-2-glycerol does not introduce significant multiplet splitting due to $^{13}$C$_{5,6}$ scalar coupling in the pyrimidine ring (Johnson et al. 2006; Hoogstraten and Johnson 2008; Dayie and Thakur 2010). Furthermore, addition of $^{13}$C-labeled sodium formate to the growth media containing unlabeled glucose leads to the selective labeling of the C8 position within the purine ring (Latham et al. 2005). We recently demonstrated that spiking the growth medium with $^{13}$C labeled formate increases the number of sites selectively enriched in K10zwf (Dayie and Thakur 2010). For cost effectiveness and selective carbon labeling, E. coli is, therefore, likely to remain an organism of choice for incorporation of isotopic labels.

Since large quantities of labeled NTPs are needed for most biophysical studies such as nuclear magnetic resonance (NMR), it is critical to find conditions that achieve optimal nucleic acid yield. Recently, Paliy and Gunasekera demonstrated that the commonly used M9 media did not support optimal growth of E. coli BL21. Rather, when ferrous sulfate was added to the M9 media and the concentration of phosphate in this media was increased to approximate the LeMaster-Richards (LMR) media (LeMaster and Richards 1982), the cell density in the stationary phase tripled (Palay and Gunasekera 2007). Studier also showed that minimal media containing 50–100 mM phosphate was optimal for protein and plasmid production (Studier 2005). Based on these observations, we hypothesize that the Studier and LeMaster-Richards but not M9 media will be optimal for nucleic acid production.

Therefore, in this work we characterize the growth of three E. coli strains in three different minimal media for production of specifically labeled nucleotides: M9, LMR, and Studier phosphate buffer (SPG; LeMaster and Richards 1982; Sambrook and Russell 2001; Studier 2005). We find that both the LMR and SPG media outperform the traditional M9 media, and growth of E. coli in both LMR and SPG provide similar amounts of genomic DNA and RNA for subsequent hydrolysis to nucleoside monophosphates. In turn, the nucleosides triphosphates can be used to transcribe labeled RNA and DNA of any desired sequence.

### Material and methods

**Bacterial strains**

The strains used in this work were obtained from the following sources: Dr. Paliy kindly provided the wildtype K12 NCM3722 (Soupene et al. 2003); the Coli Genetic Stock Center (CGSC) was the source of the wildtype K12 strain (Clowes 1968) (CGSC # 4401:F$^+$), the mutant strain DL323 (LeMaster and Kushlan 1996) (CGSC # 7538: F$^-$ mdh-1, mufA22, zwf-2, relA1, T$_2$R, pfk-$^b$, pyk-10).
Isotopes

All labeled compounds were purchased from Cambridge Isotope Laboratory (Andover, MA, USA) and Isotec-Sigma Aldrich (Miamisburg, OH, USA) with the following isotopic enrichments: sodium $^{13}$C-2-acetate (99%), $^{13}$C-2-glycerol (99%), $^{13}$C-1,3-glycerol (99%), sodium $^{13}$C-formate (99%) and $^{15}$N-$\text{(NH}_4\text{)}_2\text{SO}_4$ (99%).

Stock solutions

The stock solutions required for bacterial growth were prepared using distilled and deionized heat-sterilized water. The 10,000× trace metals solution was prepared in 10 ml aliquots containing 5 mL of 0.1 M FeCl$_3$·6H$_2$O dissolved in ~0.1 M HCl, 0.2 mL of 1 M CaCl$_2$, 0.1 mL of 1 M MnCl$_2$·4H$_2$O, 0.1 mL of 1 M ZnSO$_4$·7H$_2$O, 0.1 mL of 0.2 M CoCl$_2$·6H$_2$O, 0.2 mL of 0.1 M CuCl$_2$·2H$_2$O, 0.1 mL of 0.2 M NiCl$_2$·6H$_2$O, 0.2 mL of 0.1 M Na$_2$MoO$_4$·5H$_2$O, 0.2 mL of 0.1 M Na$_2$SeO$_3$·5H$_2$O, 0.2 mL of 0.1 M H$_3$BO$_3$, FeSO$_4$·7H$_2$O (Tyler et al. 2005). All stock solutions of individual metals except acidified FeCl$_3$·6H$_2$O were heat-sterilized and the trace metals solutions were wrapped in aluminum foil and stored at 25°C until use.

Media for bacterial growth

Luria Bertani, M9, LMR, and SPG minimal media were prepared as described (LeMaster and Richards 1982; Sambrook and Russell 2001; Paliy et al. 2003; Studier et al. 2005) with compositions shown in Supplementary Table 1. For the optimization step, each media was supplemented with varying (0.05–5% w/v) carbon source of sodium acetate, glycerol, glycerol combined with sodium formate, and varying (0.01–5% w/v) nitrogen source of ammonium sulfate. We are interested in finding optimal conditions for growth in acetate because labeled acetate can be used optimally for uniform labeling using K12. However, K12 is not optimal for site-specific labeling using glycerol and formate because of scrambling of the label in the ribose and nucleobase moieties. However, K10zwf and DL323 are ideal for site-specific labeling and so we present data for these two strains using either glycerol alone or glycerol with formate. But for K12, we present data for only acetate.

Optimization of growth conditions

The growth of each bacterial strain was optimized for the highest production of biomass per input gram of carbon source by growing 10 and 500 ml cultures with increasing amounts of each carbon source.

For 5–10 ml starter cultures of each E. coli strain (DL323, K10zwf, K12), aliquots from glycerol stocks stored at $-80^\circ$C were grown overnight at 270 rpm and 37°C in SPG media containing the following: 25 mM (NH$_4$)$_2$SO$_4$, 50 mM KH$_2$PO$_4$, 50 mM Na$_2$HPO$_4$ and 2 mM MgSO$_4$. This SPG media was supplemented with 0.4% w/v glycerol and 0.2× trace metal solution. The overnight culture was pelleted at 3,000 rpm at 4°C for 5 min, the pellets were washed twice with 1× phosphate-buffered saline and then gently resuspended in 10 ml of SPG media. About 50 μl of the dissolved pellet was added to each of the conditions tested in a total volume of 10 ml. By starting with an OD$_{600}$ of 0.05 for all resuspended pellets, highly reproducible results with consistent lag times were obtained. Unless noted otherwise, at least three independent experiments were carried out for all three strains in all growth conditions tested.

Results

RNA is estimated to be 20% of the dry weight of rapidly dividing E. coli cells (Feist et al. 2007). By isolating total nucleic acids from bacterial cells grown on isotopically labeled precursors, labeled monomers needed for making RNA of any desired sequence can be synthesized in very large quantities. Particularly in the area of RNA biophysics, custom made nucleotides that are site-selectively enriched with $^{13}$C and/or $^{15}$N stable isotopes are desirable for tackling the structures of large RNAs (Dayie 2008; Lu et al. 2010). Therefore, the judicious choice of E. coli strains and labeled precursors is important in achieving those goals.

Previous work by Hoffman and Holland (1995) and Nikonowicz (2001) established that wildtype E. coli strains grown on acetate produce labeled nucleotides useful for making RNA for NMR studies. Recent work by Hoogstraten et al. also demonstrated that glycerol is a useful complement to acetate for site-specific labeling of nucleotides for biophysical studies using mutant E. coli strains (Johnson et al. 2006; Johnson and Hoogstraten 2008). Given the high cost of most labeled precursors, it is important to obtain optimal growth conditions for these E. coli strains in various commonly used media.

Thus to ascertain optimal growth conditions, we grew E. coli K12-NCM3722 (Soupene et al. 2003), K10zwf (Fraenkel 1968), and DL323 (LeMaster and Kushlan 1996) in three different minimal media supplemented with two different carbon sources.

K12 strain grew well on glycerol with a lag phase of ~1 h in all three media (LMR, SPG, and M9) followed by normal S-shaped growth (Table 2, Fig. 1) and a doubling time of ~1.3–1.7 h. In addition, the maximum cell optical density was...
slightly higher in LMR and SPG (∼2.4) than in M9 (∼1.9) media. With acetate as the sole carbon source in M9, the growth of K12 is reduced to ∼0.6 times the maximum cell optical density observed for the growth in glycerol. The doubling time remains comparable to that observed in glycerol for LMR and SPG media, yet it is ∼2–3 times the growth observed in acetate M9 media (Table 2). Furthermore, the lag phase quadruples when using acetate as the carbon source compared to glycerol for all three media. These observations are similar to those observed in glycerol for LMR and SPG media.
reported for the growth of K12 in acetate media that also exhibited a lag time of $\sim 5$ h and a doubling time of $\sim 3$ h (Paliy and Gunasekera 2007). Thus, the growth of K12 in M9 media appears crippled compared to growth in SPG and LMR media using either glycerol or acetate as the sole carbon source.

Similar to K12, the K10zwf strain grew well on glycerol with lag phase of $\sim 1$ h in all three media (LMR, SPG, and M9) followed by normal S-shaped growth (Table 2, Fig. 1) and a doubling time of $\sim 1.4$ h. The maximum cell optical density was slightly higher in LMR and SPG (1.9–2.2) than in M9 (1.5) media. As observed with the growth of K12 in...
acetate M9, the growth of K10zwf is reduced to ~0.7 times the maximum cell optical density observed for the growth in glycerol. Again, the doubling time for growth in acetate is ~1.3–2 times that in glycerol (Table 2), and the lag phase is two to three times greater in acetate compared to that in glycerol for all three media. In agreement with our observations, previous studies on K10zwf, mostly done in glucose, indicate that the doubling time is comparable to what is observed in glycerol, but almost two times faster than what is observed in acetate (Table 2).

DL323 also grew considerably well on glycerol with a lag phase of ~1 h and doubling time of ~1.5 h in all three media (LMR, SPG, and M9) followed by normal S-shaped growth (Fig. 1). Unlike K10zwf and K12, DL323 did not grow in acetate-based M9 media for up to 30 h. On the whole, the growth of DL323 in glycerol is similar in lag phase and doubling time to that of K12 and K10zwf in LMR and SPG, but this growth is completely impaired in acetate M9 buffer (Fig. 1, Table 2).

In addition to characterizing how well each E. coli strain grows in various media, an equally important aspect of working out optimal conditions for growth is finding the required amount of carbon and nitrogen sources needed for favorable growth. The growth of all three E. coli strains were optimized by growing 500 ml cultures containing varying acetate concentrations of 0.05–5% w/v and glycerol concentrations of 0.1–10% w/v (in triplicate), as shown in Fig. 2. The optimal growth for K12 appears centered on 0.3–1% w/v acetate. For K10zwf and DL323 this growth is centered on 0.2% w/v glycerol. While this optimum falls sharply for the growth of K12 in acetate, it is slightly broader for DL323 and K10zwf grown in glycerol (Fig. 2a). K10zwf and DL323 strains grow in sodium formate (in a glycerol background) with broad optima of ~0.01–0.25% w/v; so that a spike of ~0.02% sodium formate is sufficient to support growth in K10zwf and DL323 (Fig. 2b). Finally, optimization of the amount of nitrogen source using 0.05–0.5% w/v for (NH₄)₂SO₄ indicated that below 0.1% (NH₄)₂SO₄ the growth of K10zwf and DL323 on SPG glycerol media was slightly limiting, and both strains achieve a broad growth optimum centered on 0.1–0.3%. For this work, 0.2% was selected. For K12 grown in SPG acetate media, this optimum increases only slightly with ammonium ion concentration over the ranges tested (Fig. 2c).

The maximum optical cell density is a good indicator of overall cell growth, but it does not directly report on the amount of ribonucleoside monophosphates made, which is the ultimate goal of growing cell cultures to make labeled ribonucleotides. To ascertain how much of the cell mass is ribonucleotides, we performed a nuclease P1 digest monitored by UV, agarose denaturing gel, and direct carbon and two-dimensional NMR spectroscopy. For some of the growth conditions discussed previously, the ratio of the rNMP/dNMP was quantified (Table 3). Glycerol-based LMR media achieves a slightly higher cell mass density than glycerol-based SPG media for the growth of DL323 (Fig. 1c) and the corresponding yield of ribonucleotides are also lower for growth in the glycerol-based SPG media (Table 3). Not surprising then, the ratio of the rNMP:dNMP is higher for LMR compared to SPG media for growth of E. coli DL323. The growth of K12 in SPG acetate media gives the lowest yield of 1.6 g of wet weight biomass and the lowest rNMP:dNMP ratio of less than 2. K10zwf, on the other hand, had the highest rNMP:dNMP ratio of 6.7–7.2 and yield of total rNMPs of 50–67.8 mg/L culture in SPG glycerol media.

**Discussion**

For many biophysical analyses of biomolecules, milligram quantities of these biopolymers are frequently needed. Particularly, the ability to make RNA and proteins labeled with ¹³C and ¹⁵N isotopes is critical for solving the solution structures and probing the dynamics and binding of RNAs and proteins. To synthesize the milligram quantities of labeled materials required for such work, the cost involved can become prohibitive. The cost of labeled carbon is the major component of the overall cost for ¹³C/¹⁵N labeling. Therefore, we have evaluated the conditions under which some of the commonly used E. coli strains (K12, K10zwf, and DL323) grow optimally in three minimal media compositions with isotopic-labeled carbon sources of acetate, glycerol, and glycerol combined with formate. Our results substantiate our hypothesis that both the LMR and the SPG media surpass the commonly used M9 media, and both support growth of E. coli in the production of nucleotides. Compared to glycerol, growth in acetate media is substantially reduced.

The overall diminished growth in acetate compared to that in glucose or glycerol is not unexpected given many of the genes correlated with growth rate in the cell are substantially downregulated in E. coli grown in acetate as the sole carbon source (Fig. 3; Oh et al. 2002).

The growth characteristics of the zwf mutant are comparable to the K12 wildtype and can be rationalized similarly by consideration of the central metabolic pathway in E. coli. In the zwf mutant, the glucose-6-phosphate dehydrogenase gene is deleted and the activity of the enzyme is not detectable in crude cell-free extracts (Zhao et al. 2004). Interestingly, even though the carbon flux through the oxidative pentose phosphate pathway to the non-oxidative pentose phosphate pathway (no-PPP) is blocked in the zwf mutant, this flux is re-routed through the reverse no-PPP. Hence, relative to the wildtype, zwf mutants grown on glucose direct 20% more carbon flux through the first step of the Embden–Meyerhof pathway and about 14–26% more
The carbon flux increases for the early steps of gluconeogenesis (Zhao et al. 2004). The overall effect of this carbon flux redistribution is that the zwf mutation does not adversely affect cell growth but grows comparably as the wildtype, in agreement with our results here.

The growth characteristics of the sdh/mdh mutant (DL323) on glycerol are comparable to that of the wildtype and the zwf mutant, but growth on acetate M9 is weakened compared to wildtype and the zwf mutant. This can also be rationalized as follows. Disruption of the TCA cycle in the double mutant of malate dehydrogenase and succinate dehydrogenase should eliminate the fraction of oxaloacetate generated through the TCA cycle. The anaplerotic PEP carboxylase is then expected to exclusively generate oxaloacetate (LeMaster and Kushlan 1996; Fischer and Sauer 2003). For growth on acetate as the sole carbon source, the glyoxylate shunt is the only pathway allowing growth on acetyl Co-A (Cozzone 1998). In the absence of sdh, succinate cannot be converted to fumarate, and therefore malate. In turn, malate cannot be converted to oxaloacetate in the absence of mdh. In addition, the PEP carboxylase expression levels are downregulated in acetate (Oh et al. 2002). Consequently, sdh/mdh mutants should barely grow on acetate. This is in agreement with our observations.

Labeling patterns useful for NMR applications are obtained in the aromatic ring of NMPs derived from K12 bacterial cultures, and yet the labeling pattern within the ribose ring is less desirable because adjacent carbon sites...
are labeled in the ribose ring (Hoffman and Holland 1995). The large scalar J-couplings within these labeled sites lead to unwanted reduction of resolution and signal intensity in NMR experiments. Some of these undesirable labeling patterns are absent in NMPs derived from K10zwf bacterial cultures (Johnson et al. 2006; Dayie and Thakur, 2010). Yet until now, it was unclear how well K10zwf grows on acetate or glycerol. Previous studies were carried out using mostly glucose and acetate (Fischer and Sauer 2003; Zhao et al. 2004; Nicolas et al. 2007).

The NMPs derived from K10zwf cultures do not have some of the deficiencies of those obtained from K12. Nonetheless, the aromatic moiety of the NMPs extracted from K10zwf and K12 cultures still suffer from the scrambling of isotopic label in the aromatic ring due to the efficiency of the TCA cycle. E. coli strain DL323, deficient in the two TCA cycle enzymes sdh and mdh, limits the dilution of labeling via the TCA cycle. Again, until now, the extent to which DL323 E. coli cells grow on acetate or glycerol had not been fully characterized. The studies reported herein should therefore benefit those interested in harnessing the potential of these E. coli strains to make selective labels for NMR structural studies.

In summary, growth of the three E. coli strains K10zwf, DL323 and K12 in glycerol is best done in LMR whereas growth of K10zwf and K12 in acetate is best done in either LMR or SPG media. M9 is not recommended for any of the E. coli strains tested in either glycerol, glycerol and formate, or acetate. One can obtain 24–66 mg of total $^{13}$C-labeled rNMPs, per 1,000 ml culture, which is sufficient to make labeled NMR samples at a cost of $200$–$400 per sample. E. coli is likely to remain an organism of choice for incorporation of isotopic labels for making labeled RNA for biophysical studies.

Fig. 3 Prediction of expression levels of central metabolic pathway genes in glycerol and acetate derived from gene expression profiling and metabolic flux data (Oh and Liao 2000; Oh et al. 2002) and from protein expression data (Peng and Shimizu 2003). Red (dotted) lines represents upregulation of individual genes in either glycerol or acetate grown cells relative to glucose grown cells. The X represent mutations of E. coli strains ( zwf: K10-1516; and mdh and sdh: DL323) used in this study. a The relative expression levels of the central metabolic pathway genes in glycerol relative to those in glucose. For example the expression levels of pykF, pykA and mdh are high. b The relative expression levels of the central metabolic pathway genes in acetate relative to those in glucose. The expression levels of ppsA, maeB, ppc, malate synthase, acs, mdh were up-regulated. G6P glucose-6-phosphate, F6P fructose-6-phosphate, DHAP dihydroxyacetone phosphate, G3P glyceraldehyde-3-phosphate, TKL transketolases, TAL transaldolases, F6P fructose-6-phosphate, E4P erythrose-4-phosphate, S7P septulose-7-phosphate, R5P ribose-5-phosphate, Ru5P ribulose-5-phosphate, X5P xylose-5-phosphate, 2-KDPG 2-keto-3-deoxygluconate-6-phosphate, PEP phosphoenolpyruvate, OAA oxaloacetate, GLX glyoxylate, PYR pyruvate, AcCoA acetyl-coenzyme A, CIT citrate, ICT isocitrate, 2-KG 2-keto glutarate, SUC succinate, FUM fumarate, MAL malate, EMP pathway Embden–Meyerhoff–Parnas pathway, ED pathway Entner–Doudoroff, pyk pyruvate kinase, ppsA phosphoenolpyruvate synthetase, mdh malate dehydrogenase, sdh succinate dehydrogenase, acs acetyl co-enzymeA synthetase, ppc phosphoenolpyruvate carboxylase, pckA phosphoenolpyruvate kinase
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