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# Growth of *E. coli* BL21 in minimal media with different gluconeogenic carbon sources and salt contents

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**Abstract** *Escherichia coli* strain BL21 is commonly used as a host strain for protein expression and purification. For structural analysis, proteins are frequently isotopically labeled with deuterium ($^2$H), $^{13}$C, or $^{15}$N by growing *E. coli* cultures in a medium containing the appropriate isotope. When large quantities of fully deuterated proteins are required, *E. coli* is often grown in minimal media with deuterated succinate or acetate as the carbon source because these are less expensive. Despite the widespread use of BL21, we found no data on the effect of different minimal media and carbon sources on BL21 growth. In this study, we assessed the growth behavior of *E. coli* BL21 in minimal media with different gluconeogenic carbon sources. Though BL21 grew reasonably well on glycerol and pyruvate, it had a prolonged lag-phase on succinate (20 h), acetate (10 h), and fumarate (20 h), attributed to the physiological adaptation of *E. coli* cells. Wild-type strain NCM3722 (K12) grew well on all the substrates. We also examined the growth of *E. coli* BL21 in minimal media that differed in their salt composition but not in their source of carbon. The commonly used M9 medium did not support the optimum growth of *E. coli* BL21 in minimal medium. The addition of ferrous sulphate to M9 medium (otherwise lacking it) increased the growth rate of *E. coli* cultures and significantly increased their cell density in the stationary phase.

**Keywords** *Escherichia coli* · BL21 · Gluconeogenesis · Minimal medium · Acetate · Succinate

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## Introduction

*Escherichia coli* is commonly used as a host for protein expression as it constitutes a simple and well-studied system and provides a wide choice of expression options (Bhandari and Gowrishankar 1997). *E. coli* strain BL21 has defective *lon* and *ompT* proteases and therefore is well suited for protein overexpression (Yadava et al. 2005). This strain has been used extensively to express a variety of native and heterologous proteins (Sosa-Peinado et al. 2000; Zhong et al. 2005; Gilsdorf et al. 2006; Lee and Keasling 2006). For many methods of protein analysis, large quantities of protein are often required and, therefore, the ability to achieve the maximum protein yield from a specific culture volume is important (Vanatalu et al. 1993; Sosa-Peinado et al. 2000; Zhong et al. 2005).

Labeling with stable isotopes such as deuterium (D=$^2$H) is a powerful tool for studying the functional structure of biomolecules by nuclear magnetic resonance spectroscopy and neutron scattering (Gardner and Kay 1998; Tuominen et al. 2004). In both methods, protons and deuterons produce different types of signal; selective deuteration therefore provides contrast (LeMaster 1990; Gardner and Kay 1998). To obtain proteins enriched in deuterium, they are often overexpressed in *E. coli* cells such as BL21 grown in $D_2O$-based minimal medium supplemented with a completely deuterated carbon source (Gardner and Kay 1998; Sosa-Peinado et al. 2000). Among these deuterated carbon sources, deuterated succinate and acetate are less expensive than deuterated glucose or deuterated rich media such as algal hydrolysate (prices from Cambridge Isotope Laboratories, 2005), and thus *d*-succinate or *d*-acetate might be advantageous to use for large-scale protein production (LeMaster and Richards 1988; Vanatalu et al. 1993; Paliy et al. 2003). Although the glucose utilization pathways have been recently investigated for *E. coli* BL21 strain (Phue and

Shiloach 2004; Phue et al. 2005), the growth in gluconeogenic carbon sources has not been extensively studied. In this study, we characterized the growth of *E. coli* BL21 on different gluconeogenic carbon sources and in different minimal media. We also compared BL21 growth with that of K12 and JM109 strains.

## Materials and methods

The *E. coli* strains used were wild-type K12 strain NCM3722 (Soupene et al. 2003), JM109 (New England Biolabs; F' *traD36 proA*$^+$*B*$^+$*lacI*$^q$ Δ(lacZ)M15/Δ(lac-proAB) glnV44 e14$^-$ *gyrA96 recA1 relA1 endA1 thi hsdR17*), BL21 (Amersham Biosciences; F$^-$ *ompT [lon] hsdS*$_B$ ($r_B^-$ $m_B^-$) *gal dcm*), BL21 [DE3] (Novagen; F$^-$ *ompT [lon] hsdS*$_B$ ($r_B^-$ $m_B^-$) *gal dcm* [DE3]), and BL21 [DE3] Rosetta pLysS (Novagen; F$^-$ *ompT [lon] hsdS*$_B$ ($r_B^-$ $m_B^-$) *gal dcm lacY1* [DE3] pLysSPARE [*argU, argW, ileX, glyT, leuW, proL; Cm*$^R$]).

Luria–Bertani and M9 minimal media were prepared and used as described (Sambrook and Russell 2001). LeMaster and Richards minimal medium (LR medium) consisted of a mixture of buffer salts ($KH_2PO_4$ 24.0 g l$^{-1}$ and NaOH 1.0 g l$^{-1}$), and mineral salts [$(NH_4)_2SO_4$ 1.675 g l$^{-1}$, $MgSO_4 \cdot H_2O$ 0.3 g l$^{-1}$, $FeSO_4 \cdot 7H_2O$ 3.0 mg l$^{-1}$, concentrated $H_2SO_4$ 10 μl l$^{-1}$], prepared as 10× and 100× concentrates, respectively, and mixed as buffer salts–mineral salts–water in the ratio 10:1:89 (LeMaster and Richards 1982; Paliy et al. 2003). N$^-$C$^-$ minimal medium was as described (Gutnick et al. 1969); as a source of nitrogen, 10 mM ammonium chloride was used. Each medium was supplemented with an appropriate amount of a carbon source. The carbon sources used in this study were glucose ($C_6H_{12}O_6$) (0.20% *w/v*), glycerol ($C_3H_8O_3$) (0.20%), sodium pyruvate ($C_3H_3O_3Na$) (0.24%), sodium succinate ($C_4H_4O_4Na_2 \cdot 6H_2O$) (0.45%), sodium acetate ($C_2H_3O_2Na$) (0.27%), malic acid ($C_4H_6O_5$) (0.22%), sodium fumarate ($C_4H_2Na_2O_4$) (0.27%), and oxaloacetic acid ($C_4H_4O_5$) (0.22%). The carbon sources were added to minimal medium in the above amounts to achieve an equal concentration of carbon atoms in each medium. For the comparison of different minimal media (M9, N$^-$C$^-$, and LR), 0.4% glycerol was used as a carbon source. The carbon sources were sterilized separately and then added to minimal media adjusted to pH=7.0.

*E. coli* cultures were first grown aerobically at 37 °C in M9 medium supplemented with 0.2% glycerol. The cells were harvested by centrifugation, washed twice with phosphate-buffered saline (0.15 M, pH 7.3±0.2), and resuspended to the desired starting optical density (OD) in appropriate fresh medium. Inoculated cultures were grown aerobically in a water bath shaker (220 rpm) in either Erlenmeyer flasks (medium/flask volume—1/10) or test tubes (initial culture volume—10 ml) at 37 °C until they reached a stationary phase. Growth was monitored spectrophotometrically by measuring each culture's $OD_{600nm}$ periodically. Unless otherwise stated, three independent experiments were carried out for the growth of BL21 strain in each medium.

## Results

We grew *E. coli* BL21 [DE3] strain (Novagen) aerobically on a variety of carbon sources in M9 minimal medium. This strain grew reasonably well on glucose (data not shown), glycerol, and pyruvate (Fig. 1). However, the cultures showed no increase in their OD values on succinate and acetate in the first 20 and 10 h of cultivation, respectively. After the long lag-phase, growth proceeded normally as was evidenced by the typical S-shaped growth curves of the cultures (Fig. 1), though the final cell densities of acetate and succinate cultures were lower than that for glycerol-grown cells (final $OD_{600nm}$ of 0.56±0.06, 0.60±0.02, and 0.86±0.02 for succinate, acetate, and glycerol, respectively). We have used plate colony counts of diluted BL21 cultures to confirm that the long lag-phase of BL21 cultures on acetate and succinate was due to the physiological adaptation of *E. coli* cells to the new carbon source (cells were pre-grown in glycerol-based minimal medium) and that all or most of the cells were able to grow on these carbon sources after the adaptation period (see

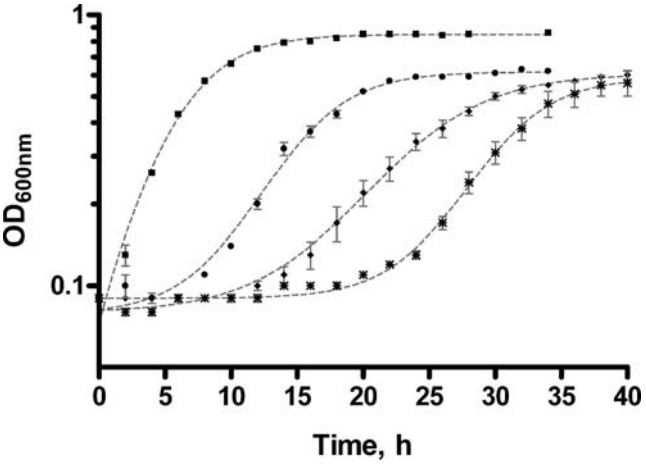


**Fig. 1** Growth of *E. coli* BL21 on different carbon sources in aerobic batch cultures. The M9 medium was supplemented with 0.20% glycerol (*filled squares*), 0.24% pyruvate (*filled circles*), 0.45% succinate (*asterisks*), and 0.27% acetate (*filled diamonds*). The amounts of carbon sources were added so as to achieve an equal number of carbon atoms in each medium. Data points are shown as averages with *error bars* representing the range of OD values for each time point. In many cases, the error bars are very close to the average data point and, therefore, are not visible. Each *line* represents a sigmoidal fit of the corresponding growth data

supplementary material at http://www.med.wright.edu/bmb/op/papers/BL21). In contrast to *E. coli* BL21, wild-type NCM3722 (K12) grew well on all of the carbon sources tested, with only the acetate cultures showing a modest lag phase of 5 h (http://www.med.wright.edu/bmb/op/papers/BL21). The growth rate of cultures in each medium (and in many cases the final $OD_{600}$ reached) was lower for BL21 than for NCM3722 (http://www.med.wright.edu/bmb/op/papers/BL21). Although it is well known that *E. coli* grows more slowly on gluconeogenic carbon sources than on glucose (Andersen and von Meyenburg 1980), we could not find any reports of the growth problems of *E. coli* BL21 in minimal medium supplemented with succinate or acetate. Novagen, from whom the initial BL21 strain was purchased, was also not aware of such growth deficiencies (personal communication). To test the growth of BL21 on gluconeogenic carbon sources further, we also grew this strain on fumarate, malic acid, and oxaloacetate. BL21 grew well on malate and oxaloacetate but had an extended lag-phase on fumarate; the growth curve of the fumarate-based cultures was similar to that for succinate-grown bacteria (Fig. 1; http://www.med.wright.edu/bmb/op/papers/BL21). NCM3722 grew well on all these carbon sources. *E. coli* strain JM109, used frequently for genetic engineering studies, also grew on all carbon sources, with a long lag-phase only observed in acetate-based medium (http://www.med.wright.edu/bmb/op/papers/BL21). The observed growth behavior of BL21 was not limited to the BL21 [DE3] strain acquired from Novagen. Both BL21 from Amersham Biosciences and Rosetta BL21 [DE3] pLysS from Novagen showed similar growth patterns (data not shown).

We also examined the aerobic growth of BL21 in different minimal media. Three different minimal media were used: M9 minimal medium, commonly used in microbiological studies; $N^-C^-$ minimal medium, utilized for physiological experiments and gene expression studies (Soupene et al. 2003; Gyaneshwar et al. 2005); and LR medium, used for preparation of deuterated components in *E. coli* (LeMaster and Richards 1982; Paliy et al. 2003). The medium recipes differed in the amounts of inorganic ions but not in the source of carbon (0.4% glycerol was used in each case). Compared to $N^-C^-$ and M9 media, LR medium allowed BL21 cultures to achieve greater final cell density and to maintain substantially better growth rates in the exponential phase of growth (Table 1). The results were independent of the source of water (deionized vs distilled) used for medium preparation. Similar differences between M9 and LR media were observed for the growth of NCM3722 strain (data not shown). When medium recipes were compared, the observed growth differences were thought to be attributed to the presence of bivalent iron and to the higher phosphate content of the LR medium. When M9 medium was supplemented with ferrous sulphate or additional potassium phosphate ($FeSO_4 \cdot 7H_2O$ and $KH_2PO_4$ were added, respectively) so as to achieve the concentrations present in the LR medium, BL21 strain grew well, with a growth rate and final $OD_{600}$ values substantially better than those for the original M9 medium (Table 1).

## Discussion

*E. coli* strain BL21 had a prolonged lag-phase during aerobic growth on succinate, acetate, and fumarate as the carbon source in M9 minimal medium, though it grew well on glycerol, pyruvate, oxaloacetate, and malate. It has been recently reported (Phue and Shiloach 2004; Phue et al. 2005) that there was a difference in gene regulation between the BL21 and JM109 strains for some of the genes of the TCA cycle and glyoxylate shunt (*fumA*, *sdhABD*, and *acnA*). In particular, *fumA*, coding for fumarase, was expressed at a significantly lower level in BL21 than in JM109 when cells were grown in low glucose medium. The utilization of both fumarate and succinate but not acetate proceeds through *fumA* (acetate is converted into malate by enzymes of the glyoxylate shunt). This can explain the very long lag-phase of BL21 cultures on fumarate and succinate, as the initial metabolic flux through fumarase might not be sufficient to sustain growth. In contrast, acetate-grown cells displayed a considerably shorter lag-phase (see Fig. 1). It is also possible that the initial lack of growth on succinate and fumarate was due to an insufficient transport capacity for these compounds in BL21 (Boogerd et al. 1998).

We grew *E. coli* BL21 aerobically on different minimal media containing the same carbon source (glycerol). BL21 was able to grow faster and achieved a higher final cell density in LR medium. Both M9 and $N^-C^-$ media do not contain iron as it is usually present in trace

**Table 1** The maximum optical density and doubling time (DT) of *E. coli* BL21 (DE3) aerobic batch cultures in different minimal media supplemented with 0.4% glycerol

| Medium | DT (min) | Max $OD_{600nm}$ |
|---|---|---|
| M9 | 102±9 | 1.2±0.19 |
| $N^-C^-$[a] | 78±6 | 1.6±0.14 |
| LR[a] | 66±3 | 3.1±0.14 |
| M9 + $Fe^{2+}$ | 71±1 | 2.9±0.13 |
| M9 + $PO_4^{3-}$ | 71±2 | 2.2±0.08 |

$N^-C^-$ minimal medium contained 10 mM of $NH_4Cl$. $FeSO_4 \cdot 7H_2O$ (0.01 mM) and $KH_2PO_4$ (107 mM) were added to M9 minimal medium to supplement it with iron and additional phosphate, respectively. Values are shown as arithmetic mean±standard error of the mean ($n$=3).

[a] Only two replicates were used.

amounts in the water used for medium preparation. Nevertheless, the addition of bivalent iron to M9 medium increased the growth rate and the final cell densities of the BL21 cultures to the level of those grown in LR medium (iron is required by the enzymes of the tricarboxylic acid cycle and aerobic respiration chain) (Zhang et al. 2005).

When *E. coli* cultures are grown to produce large quantities of native or heterologous proteins, the cost of protein production is important. It is especially true in the case of the production of isotopically labeled proteins, as deuterated carbon sources, for example, are much more expensive than their protiated counterparts. For fully deuterated minimal media, the main contributors to the cost of the medium are the deuterated carbon source and $D_2O$. It is therefore advantageous to grow cells in the medium that achieves the highest culture yield for a given amount of deuterated carbon source. Our results indicate that LR medium is well suited for such experiments. If M9 medium needs to be used for protein expression, we suggest adding bivalent iron to the medium to enhance culture growth. We also show that despite a long lag-phase, BL21 can grow on both acetate and succinate, which are much less expensive deuterated sources of carbon. If a long lag-phase is not acceptable, glycerol or pyruvate can be used instead or the BL21 cells can be pre-adapted to growth in acetate- or succinate-based minimal media as we did previously for *E. coli* JM109 and MRE600 (Paliy et al. 2003).

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