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Replacement of the glucose phosphotransferase transport system by galactose permease reduces acetate accumulation and improves process performance of *Escherichia coli* for recombinant protein production without impairment of growth rate

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Abstract

Acetate accumulation under aerobic conditions is a common problem in *Escherichia coli* cultures, as it causes a reduction in both growth rate and recombinant protein productivity. In this study, the effect of replacing the glucose phosphotransferase transport system (PTS) with an alternate glucose transport activity on growth kinetics, acetate accumulation and production of two model recombinant proteins, was determined. Strain VH32 is a W3110 derivative with an inactive PTS. The promoter region of the chromosomal galactose permease gene *galP* of VH32 was replaced by the strong *trc* promoter. The resulting strain, VH32GalP$^+$ acquired the capacity to utilize glucose as a carbon source. Strains W3110 and VH32GalP$^+$ were transformed for the production of recombinant TrpLE-proinsulin accumulated as inclusion bodies (W3110-PI and VH32GalP$^+$-PI) and for production of soluble intracellular green fluorescent protein (W3110-pV21 and VH32GalP$^+$-pV21). W3110-pV21 and VH32GalP$^+$-pV21 were grown in batch cultures. Maximum recombinant protein concentration, as determined from fluorescence, was almost four-fold higher in VH32GalP$^+$-pV21, relative to W3110-pV21. Maximum acetate concentration reached 2.8 g/L for W3110-pV21 cultures, whereas a maximum of 0.39 g/L accumulated in VH32GalP$^+$-pV21. W3110-PI and VH32GalP$^+$-PI were grown in batch and fed-batch cultures. Compared to W3110-PI, the engineered strain maintained similar production and growth rate capabilities while reducing acetate accumulation. Specific glucose consumption rate was lower and product yield on glucose was higher in VH32GalP$^+$-PI fed-batch cultures. Altogether, strains with the engineered glucose uptake system showed improved process performance parameters for recombinant protein production over the wild-type strain.

Keywords: Aerobic acetate reduction; Glucose transport systems engineering; Recombinant *E. coli*; Fed-batch

1. Introduction

Escherichia coli is one of the most widely used hosts for recombinant protein expression. In particular, this organism is currently being used for the production of a large number of therapeutic proteins at industrial scale. During the last 20 years, there has been an increasing interest on fed-batch and high cell density cultures for the production of recombinant proteins. However, a common problem encountered in high cell concentration cultures utilized in industrial processes is the production and accumulation of acetate in the medium (Lee, 1996; Luli and Strohl, 1990). A consequence of acetate accumulation is that above a concentration of approximately 1.5 g/L, cell growth is inhibited (Bauer et al., 1990). It has also been reported that acetate accumulation impairs the capacity of *E. coli* to

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Nomenclature:

DOT	dissolved oxygen tension
q_{ac}	specific acetate production rate
q_S	specific glucose uptake rate
X	biomass concentration, measured as dry cell weight
X_{max}	maximum biomass concentration, expressed as dry cell weight
$Y_{P/S}$	product yield on glucose (for pre-proinsulin)
$Y_{X/S}$	biomass yield on glucose
μ	specific growth rate
%RP	percentage of recombinant protein (for pre-proinsulin), relative to total protein
q_F	specific fluorescence production rate

produce recombinant proteins (Jensen and Carlsen, 1990). In *E. coli*, acetate is synthesized mainly by the phosphotranscetylase-acetate kinase pathway (Pta-AckA), using acetyl coenzyme A (AcCoA) as the substrate (see Fig. 1, panel A). Acetate is produced under oxygen-limited culture conditions or during aerobic growth with a high concentration of glucose in the medium. These conditions cause an imbalance between the glycolytic and the tricarboxylic acid cycle (TCA) fluxes, resulting in the excretion of acetate and other metabolites (Majewski and Domach, 1990; Delgado and Liao, 1997; Farmer and Liao, 1997).

Since acetate accumulation is highly detrimental to *E. coli* fermentation processes, different strategies have been investigated to overcome this problem. Two general approaches have been applied to reduce acetate production in *E. coli* cultures. One is the manipulation of the culture environment to maintain either a low glucose concentration or to eliminate the accumulated acetate. Strategies such as the exponential feeding of glucose (Yoon et al., 1994), the application of control strategies based on the real-time monitoring of glucose, dissolved oxygen or acetate (Konstantinov et al., 1990; Luli and Strohl, 1990; Akesson et al., 2001), or the use of a dialysis reactor (Chalmers et al., 1990; Nakano et al., 1997) have been successful in reducing acetate accumulation in *E. coli* cultures. The second approach involves the application of metabolic pathway engineering to reduce carbon flow to the acetate-producing pathways. The elimination of Pta and AckA activities has resulted in a significant reduction in acetate accumulation (San et al., 1994). A different strategy involves the redirection of metabolic fluxes in order to reduce the metabolic availability of pyruvate for acetate synthesis (Delgado and Liao, 1997). The addition of heterologous capacities or the deregulation of endogenous biosynthetic pathways in *E. coli*, has resulted in the synthesis of low-toxicity or non-toxic metabolites, such as acetolactate instead of acetate (Aristidou et al., 1994). In addition, increasing anaplerotic flux or utilizing mutants with altered glucose transport has also resulted in reduced acetate production (Chou et al., 1994; Hosono et al., 1995; Farmer and Liao, 1997). Nonetheless, most of the molecular approaches used to date do not completely eliminate acetate production, have a deleterious effect on growth rate, or lead to undesirable pyruvate (Díaz-Ricci et al., 1991) or lactate (Yang et al., 1999) accumulation.

The direct precursor of AcCoA, pyruvate, is generated mainly by two reactions in *E. coli* growing aerobically in high glucose concentrations. One reaction is catalyzed by pyruvate kinases and the other by the phosphotransferase transport system (PTS) (see Fig. 1, panel A). The construction and characterization of strains with mutations that alter these reactions have been reported previously (Ponce et al., 1995; Flores et al., 1996; Ponce et al., 1998). Flores et al. (1996) have obtained strains with an inactive PTS that were subjected to a continuous culture selection process. PTS^- strains obtained in such a way have partially recovered their growth rate as compared to the wild-type strain (PTS^- $glucose^+$ phenotype) when using glucose as the carbon source. The characterization of these mutant strains has revealed that PTS-mediated glucose transport (see Fig. 1, panel A) was replaced by the combined action of the galactose permease (GalP) and glucokinase (Glk) (see Fig. 1, panel B) (Flores et al., 1996; Flores et al., 2002; Flores et al., 2005). Additionally, strains with both pyruvate kinase isozymes inactivated (Pyk^-) have been constructed and characterized (Ponce et al., 1995; Ponce et al., 1998). Both the PTS^- $glucose^+$ and the Pyk^- strains have been shown to direct a larger proportion of carbon flux to the aromatic pathway, when compared to an isogenic wild-type strain (Flores et al., 1996; Báez et al., 2001). Recent work has shown that these mutant strains growing in a minimal salts medium produce less acetate than a wild-type strain (Sigüenza et al., 1999).

In this work we studied the effect of inactivating only the PEP-dependent glucose transport and replacing it by an alternate ATP-dependent system composed of GalP and Glk, on the performance of *E. coli* cultures for recombinant protein production. Two culture modes and expression systems were studied: batch cultures for production of green fluorescent protein (GFP) by the wild type strain W3110-pV21 and its PTS^- derivative $VH32GalP^+$-pV21, as well as batch and fed-batch cultures for production of TrpLE-human pre-proinsulin by W3110-PI and $VH32GalP^+$-PI. GFP is a cytoplasm soluble protein induced by IPTG, whereas TrpLE-hybrid human proinsulin is accumulated as inclusion bodies induced upon tryptophan depletion. Compared to its parental strain, $VH32GalP^+$ strain showed a superior performance when producing GFP during batch cultivation, reaching higher fluorescence and importantly reducing acetate accumula-