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Inactivating FruR global regulator in plasmid-bearing *Escherichia coli* alters metabolic gene expression and improves growth rate

Dave Siak-Wei Ow [a,b,*], Raymond Ming-Yung Lee [a], Peter Morin Nissom [a], Robin Philp [a], Steve Kah-Weng Oh [a], Miranda Gek-Sim Yap [a,b]

[a] *Bioprocessing Technology Institute, 20 Biopolis Way, #06-01 Centros, Singapore 138668, Singapore*
[b] *NUS Graduate School for Integrative Sciences and Engineering, National University of Singapore, #05-01 28 Medical Drive, Singapore 117456, Singapore*

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Abstract

The introduction of plasmids into *Escherichia coli* is known to impose a metabolic burden, which diminishes the growth rate. This effect could arise from perturbation of the central metabolic pathways, which supply precursors and energy for macromolecule synthesis. We knocked out a global regulator of central metabolism, FruR (also called Cra), to assess its phenotypic effect in *E. coli* carrying plasmids. During bioreactor runs, a higher specific growth rate of 0.91 h^{-1} was observed for the plasmid-bearing fruR knockout (P+ fruR) cells compared to its parental plasmid-bearing wildtype (P+ WT) cells (0.75 h^{-1}), while both the plasmid-free cells displayed similar growth rates (1.0 h^{-1}, respectively). To investigate gene expression changes possibly related to the growth rate recovery, quantitative reverse transcriptase PCR and 2DE proteomic studies were performed. In P+ fruR cells, expression of enzymes involved in sugar catabolism, glycolysis and transcription/translation processes were upregulated, while those related to gluconeogenesis, tricarboxylic acid cycle and stress response were downregulated. Our findings demonstrate that the inactivation of FruR global regulator in recombinant *E. coli* alters metabolic gene expression and significantly reduces growth retardation from the burden of maintaining a plasmid. This study represents the first attempt to explore the role of a global regulatory gene on plasmid metabolic burden.

Keywords: Plasmid metabolic burden; Growth rate recovery; Metabolic engineering; Global transcriptional regulator; Fructose repressor; Catabolite repressor/activator

1. Introduction

Plasmids are extrachromosomal DNA vectors widely used for the production of recombinant proteins and metabolites in biotechnology. In addition, they also have potential uses in gene therapy and DNA vaccination. However, the presence of high copy plasmids can impose a metabolic burden, causing plasmid-bearing cells to grow slower than cells without plasmids (Glick, 1995). This metabolic burden is undesirable during fermentation processes as it provides a selective pressure for the formation of a plasmid-free population; and the accumulation of these non-productive cells leads to a diminished yield of the final desired product (Summers, 1991).

The central metabolic pathways of *Escherichia coli* include the major pathways of glycolysis, pentose phosphate pathway, TCA cycle, and various interlinking metabolic reactions. These pathways are the focus of metabolic engineering approaches because they supply biosynthetic precursors, energy and cofactors for both biomass and recombinant product synthesis (Holms, 1996). In bacteria, control of gene expression is coordinated by environmental and intracellular signals modulated by global regulatory proteins (Martinez-Antonio and Collado-Vides, 2003). Most regulatory proteins such as ArcAB, Crp,

Abbreviations: 2DE, two-dimensional gel electrophoresis; Cra, catabolite repressor/activator; FruR, fructose repressor; fruR cells, *fruR*::Tn5 knockout cells; LB, Luria Bertani; P+, plasmid-bearing; P−, plasmid-free; PBS, phosphate buffered saline; PTS, phosphoenolpyruvate: phosphotransferase; qRT-PCR, quantitative reverse transcriptase PCR; slpm, standard liters per minute; TCA, tricarboxylic acid; WT, wildtype

* Corresponding author at: Bioprocessing Technology Institute, 20 Biopolis Way, #06-01 Centros 138668, Singapore. Tel.: +65 6478 8859; fax: +65 6478 9561.
E-mail address: dave_ow@nus.edu.sg (D.S.-W. Ow).

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FruR, Fnr and Mlc (Perrenoud and Sauer, 2005) modulate central metabolic gene expression of *E. coli* at the transcriptional level, while CsrA (Liu and Romeo, 1997; Romeo et al., 1993) acts post-transcriptionally at the level of mRNA stability. Of these, FruR (also known as Cra) is a global regulator that directs carbon flow through the central metabolic pathways via its influence on the transcription of carbon and energy metabolism genes (Ramseier et al., 1995; Ramseier, 1996). Primarily, transcription of catabolic enzymes involved in the glycolytic pathway (pfkA, pykF, gapA, pgk, eno), Entner–Doudoroff pathway (edd, eda) and alternative sugar catabolism (fruBKA, mtlADR) are repressed, while enzymes of glyconeogenesis (fbp, ppsA), glyoxalate shunt (aceBA), TCA cycle (acnA, icdA) and electron transport chain (cydAB) are positively activated by FruR (Saier and Ramseier, 1996; Shimada et al., 2005).

We have previously reported that *E. coli* carrying a high copy plasmid showed growth retardation and global transcriptional changes in metabolic pathways (Ow et al., 2006). In plasmid-bearing cells, several FruR repressed genes in glycolysis were downregulated and other FruR activated pathways showed upregulation. This suggested that transcriptional control by FruR could be related to plasmid metabolic burden. If FruR transcriptional regulation of central metabolism is critical to the growth retardation observed during plasmid maintenance, the inactivation of FruR is expected to influence overall metabolic gene expression and cell growth. In this study, we first show a significant recovery of growth rate after inactivating FruR in plasmid-bearing *E. coli*. Subsequent qRT-PCR and 2DE proteomic analyses revealed gene expression changes related to the improved growth rate in plasmid-bearing cells.

2. Materials and methods

2.1. Bacterial strains and culturing

E. coli DH5α was the wildtype (WT) strain used. The chromosomal *fruR* gene in DH5α was disrupted by Tn5⟨KAN⟩ transposition and λ-red mediated recombination described previously (Datsenko and Wanner, 2000) to obtain *fruR*::Tn5 knockout cells (fruR cells). Plasmid-free WT and fruR cells were transformed with a 7.3 kb pcDNA3.1D/NS3 plasmid as described previously (Ow et al., 2006) to create P+ WT and P+ fruR cells, respectively. The cells were incubated overnight (37 °C, 250 rpm) in 25 ml LB media to obtain the inoculum. For shake flask cultures, 0.5 ml inoculum was inoculated in 250 ml flasks with 50 ml LB + 0.5% glucose and incubated at 37 °C and 250 rpm. OD_{600} measurements were taken hourly to determine the growth profile. For bioreactor batch cultures, 40 ml of the overnight inoculum were added into vessels containing 2 l of R25 media (12 g/l yeast extract, 6 g/l dipotassium phosphate, 6 g/l tryptone, 5 g/l arginine, 5 g/l glucose, 0.48 g/l magnesium sulfate, 0.05 g/l thiamine). Vessel temperature and pH were maintained at 37 °C and 7.0, respectively. Stirrer speed and airflow were set at 200–800 rpm and 1.5–10.0 slpm respectively, to maintain a dissolved oxygen level of more than 20%. Samples were taken every hour for OD_{600} measurement and plasmid isolation with QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). The correlation between OD_{600} and dry cell weight was determined using a standard curve to be 1 OD_{600} = 0.33 g dry cell weight/l. Glucose and acetate concentration in the supernatant were measured with YSI 7100 Multiparameter Bioanalytical System (Yellow Spring Instruments, Yellow Springs, Ohio) and acetic acid test kit (R-Biopharm, Landwehrstr, Darmstsdt), respectively. Eight milliliters of mid log cell samples (OD_{600} 1.5–2.0) were collected for the gene expression analyses.

2.2. Two-dimensional gel electrophoresis proteomic analysis

Cell samples (8 ml) were centrifuged at 4000 rpm for 10 min, washed twice with 10 ml PBS buffer before storage at −80 °C. Subsequently, the samples were lysed and 100 μg total cell proteins were loaded and separated on 2DE gel (pH 3–10 non-linear gradient). For visualization, gels were fixed and stained with the quantitative SYPRO® Orange strain as described before (Ow et al., 2006). Stained gels were scanned on a Typhoon fluorescent scanner (Amersham Biosciences, Uppsala, Sweden) and the digital images imported into PDQuest software (Bio-Rad, Hercules, CA) for analysis. Protein spots with reproducible spot intensity differences (two-tailed student *t*-test *p* value less than 0.05) between the P+ fruR and P+ WT cells were excised and identified by mass spectrometry [Applied Biosystems (Framingham, MA) QSTAR-XL hybrid quadrupole-time of flight tandem mass spectrometer (MS/MS) equipped with a nano-electrospray ionization source]. All MS/MS spectra were searched against the *E. coli* sub-set of the UniProt database (http://www.uniprot.org) using the MASCOT search engine (MatrixScience, London, England) to provide the final protein annotations.

2.3. Quantitative reverse transcriptase PCR analysis

Cell pellet samples (8 ml) were processed immediately by treatment with two volumes of RNAprotect Bacteria Reagent (Qiagen, Valencia, CA), centrifuged (4000 rpm, 10 min) and snap frozen with liquid nitrogen before storing in −80 °C freezer. RNA was extracted with Qiagen RNeasy Extraction Midi kit (Qiagen, Valencia, CA) according to the manufacturer's instruction. After elution, the RNA was treated with Promega RQ Dnase (Promega, Madison, WI) for 30 min at 37 °C. After phenol/chloroform extraction, reverse transcription was conducted with Superscript II (Invitrogen, Carlsbad, CA). The synthesized cDNA was used as the template for qRT-PCR according to the ABI SYBR Green PCR Master Mix (Applied BioSystems, Framingham, MA) protocol PN4310251D at a total volume of 25 μl. The primers used are listed in Appendix 1. PCR was conducted on ABI PRISM® 7000 Sequence Detection System (Applied BioSystems, Framingham, MA) with the following cycling conditions: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min each. Reactions were done in triplicates and relative expression changes were calculated using the ΔΔCT method (ABI Bulletin #2 PN 4303859) with the constitutively expressed rpoD gene as the endogenous control.