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Highlights

- Minimization of the metabolic burden
- Develop of an antibiotic-free expression system, devoid of resistance markers
- Improvement of the recombinant FucA production

Using promoter libraries to reduce metabolic burden due to plasmid-encoded proteins in recombinant *Escherichia coli*

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Abstract

The over-expression of proteins in recombinant host cells often requires a significant amount of resources causing an increase in the metabolic load for the host. This results in a variety of physiological responses leading to altered growth parameters, including growth inhibition or activation of secondary metabolism pathways. Moreover, the expression of other plasmid-encoded genes such as antibiotic resistance genes or repressor proteins may also alter growth kinetics.

In this work, we have developed a second-generation system suitable for *Escherichia coli* expression with an antibiotic-free plasmid maintenance mechanism based on a glycine auxotrophic marker (*glyA*). Metabolic burden related to plasmid maintenance and heterologous protein expression was minimized by tuning the expression levels of the repressor protein (LacI) and *glyA* using a library of promoters and applying synthetic biology tools that allow the rapid construction of vectors. The engineered antibiotic-free expression system was applied to the L-fuculose phosphate aldolase (FucA) over-production, showing an increase in production up to 3.8 fold in terms of FucA yield ($mg \cdot g^{-1}DCW$) and 4.5 fold in terms of FucA activity ($AU \cdot g^{-1}DCW$) compared to previous expression. Moreover, acetic acid production was reduced to 50%, expressed as $gAc \cdot gDCW^{-1}$.

Our results showed that the aforementioned approaches are of paramount importance in order to increment the protein production in terms of mass and activity.

Keywords: Synthetic Biology, Golden Gate Assembly, recombinant protein production and *Escherichia coli,* bioprocess optimization

Introduction

Among the many systems available, the gram negative bacterium *Escherichia coli* remains one of the most versatile and used host for the production of heterologous proteins, because of its rapid growth rate, the easiness to attain high cell density cultures on inexpensive substrates, its well-characterized genetics and the availability of excellent genetic tools [1]. Efforts in developing strategies to maximize the productivity of recombinant proteins in *E. coli* are well documented in the literature [2] and [3]. Extensive research has been performed over the past years in order to improve recombinant protein production in this cell factory, including the optimization of process parameters such as growth temperature, media composition, induction conditions, as well as engineering novel expression systems [4] and [5].

Recent advances in the synthetic biology, allowed the development of new methods and tools to speed up and standardize strain engineering. Compared with conventional DNA cloning protocols, these advanced DNA assembly strategies offer an efficient approach to construct multi-gene pathways in a one-step, scar-less, and sequence-independent manner. In particular, the Parts Registry is a collection of standardized biological parts (BioBricks) that allow the fast assembly of new functions [6], [7] and [8]. Individual parts or combinations of parts that encode defined functions can be independently tested and characterized in order to improve the expression system [9]. DNA construction based on the BioBrick theory has become a key part of most metabolic engineering projects and genetic circuits design. The BioBrick concept exploits the advantage that the same promoters, ribosome binding sites, expression tags, antibiotic resistances and origins of replication are frequently reused, with only the genes of interest being varied [7] and [8].

Aldolases belong to the class of lyases, which catalyze C-C bond formation leading to enantiomerically pure products, even when the starting materials are non-chiral substrates. In particular, L-Fuculose phosphate-aldolase (FucA) catalyzes the reversible reaction of L-fuculose-1-phosphate to dihydroxyacetone phosphate (DHAP) and L-lactaldehyde *in vivo*. *E. coli* has been proven to be an efficient platform for soluble overexpression of a wide range of aldolases, both endogenous and from other bacteria [10] and [11].

Vidal et al. [12] used rhamnulose 1-phosphate aldolase (RhuA) as a model protein to develop an auxotrophic marker-based expression system consisting of the M15Δ*glyA* strain, with a genome deletion of the *glyA* gene, and a two-plasmid system using the commercial pQE-40 (Qiagen) expression vector, which uses the stronger T5 promoter [13]. The *E. coli glyA* gene encodes for the enzyme serine hydroxymethyl transferase (SHMT), which catalyzes the reversible interconversion between L-threonine and glycine and between serine and glycine [14]. Although previous studies have shown that the auxotrophic *glyA*-based expression vector is a promising alternative approach to the use of antibiotic selection markers [13], increased SMHT levels leads to a metabolic burden, which causes a decrease in activity and specific productivity of recombinant proteins compared to the original system. Besides, the presence of a metabolic load generally brings to a decreased level of energy available for a variety of cellular functions, i.e. for cell maintenance and growth.

On the other hand, the use of a two-plasmid expression system often requires the presence of their respective antibiotic markers and this fact is a limitation for the production of certain compounds of pharmaceutical or clinical interest. In our case study, it is of paramount importance to fine-tune the *glyA* and *lacI* expression levels and to eliminate the pREP4 plasmid in order to overcome these limitations and allow the development of an antibiotic-free expression system.

In this work, the FucA aldolase has been used as a model protein and its gene (*fucA*) has been firstly cloned into the Qiagen commercial expression system in order to obtain high intracellular expression levels. Secondly, through the application of different synthetic biology approaches, the design and construction of an M15/pQE40-derived expression system consisting of a single vector is presented. Thirdly, the expression levels of the key genes *lacI* and *glyA* have been tuned by the use of different constitutive promoters. Finally, to completely avoid the presence of the antibiotic resistance gene, considered unacceptable in many areas of biotechnology by regulatory authorities [15], the expression system has been further engineered to be finally devoid of antibiotic resistance marker genes and tested for FucA production in shake flasks.

Materials and methods

Bacterial strains

The bacterial strain K12-derived *E. coli* M15 (Qiagen) and M15*ΔglyA* were used for recombinant FucA expression. The strain *E. coli* DH5α was used for plasmid construction and propagation. The strains were stored at -80°C in cryo-stock aliquots prepared from exponential phase cultures grown in Luria-Bertani (LB) medium. Bacterial strains used in this study are summarized in Table S1 (Supplemented materials). While, abbreviation for all the *E.coli* strains used are summarized in Table S2 (Supplemented materials).

Molecular biology techniques

Plasmid and strain constructions

Plasmid DNA and DNA fragments were isolated or purified using PreYield™ plasmid miniprep system and Wizard® SV gel and PCR clean-up system (Promega) according to the manufacturer's instructions. Restriction enzymes were purchased from Thermo Scientific and T4 DNA ligase from Roche. Transformation of *E. coli* DH5α, M15 and M15*ΔglyA* competent cells with the DNA ligation reactions was performed by electroporation using a GenePulser MXcell™ electroporator from Bio-Rad, with a pulse (V=2500v; C=25 µF; R=200 Ω). Transformants were grown on LB-agar medium or on defined medium (DM)-agar plates supplemented with antibiotic, ampicillin and kanamycin with a final concentration of 100

mg·L^{-1} while the chloramphenicol 30 mg·L^{-1}). Transformant clones were confirmed by colony-PCR, single or double restriction digests and DNA sequencing.

PCR reactions

For fragments up to 2.0 Kb, KOD Polymerase Novagen from Merck Biosciences was used whereas fragments >2.0 Kb, Phusion high-fidelity DNA polymerase (Thermo Scientific) was used, following the guidelines provided by the manufacturer, respectively. For the verification of ligation reactions and transformations, colony PCR was performed using the GoTaq® master mix (Promega). Primers are listed in Table S2 (supplemented materials).

Four promoters used (J23117, J23100, J23111 and J23100) in this study and were selected from a combinatorial library of constitutive promoters (Registry for Standard Biological Parts, http//parts.igem.com). The strength of the promoters is calculated as the reported activities of red fluorescence protein, being the J23117 the reference promoter, that is, with a given relative transcription efficiency of 1 (in arbitrary units). The promoters J23110, J23111 and J23100 are 5.2, 9.2 and 15.7 fold strongest than J23117, respectively. Each promoter was synthetized by oligonucleotide hybridization including two *Bsa*I sites with 2 different overhangs at both, 5' and 3' terminus.

PCR, agarose gel electrophoresis and DNA sequencing were performed to check all the cloning reactions following routine protocols as described in Green and Sambrook [16].

Plasmid constructs

Plasmids used in this study are summarized in table S1 (Supplemented materials).

pQE-FucA

The commercial vector pQE-40 (Qiagen) was used as reference vector for the expression of the protein of interest, namely FucA. This expression vector is based on the IPTG-inducible T5 promoter, derived from the T5 phage. This promoter is recognized by *E. coli* RNA polymerase, and has a double *lac* operator (*lac* O) repression module in series to provide tightly regulated and high-level expression of recombinant proteins (Figure S1A). The *fucA* gene was amplified from the pTrc*fuc* vector using the FucA_FW and FucA_REW primers (Supplementary materials table S2). Thereafter, the 0.65 Kbp PCR fragment was digested with the restriction enzymes *Bam*HI and *Hin*dIII and subsequently cloned into the linearized pQE-40, yielding pQE-FucA (Figure S1B). The final product was then transformed into *E. coli* M15 [pREP4] cells.

pQEαβFucA

The DNA fragment comprising the *glyA* gene was amplified from pQEαβRham (designated as αβTerminator) [13], including the promoter and the 3' termination region. The resulting 1,784 Kbp

fragment was then digested with *Bsp*EI and *Xba*I and subsequently ligated into pQE-FucA to obtain pQEαβFucA (Figure S1C). The ligation reaction was transformed into *E. coli* M15Δ*glyA*[pREP4].

BioBrick-based vectors (pSB1C3-J231XX-*lacI-glyA*)

The BioBrick vectors were assembled using the golden gate technique [17]. This strategy exploit the ability of type IIs endonucleases to cleave DNA outside the recognition site leaving an overhang sequence. In this study *Bsa*I restriction enzyme and four nucleotide overhangs is used.

Both the *lacI* and *glyA* genes were PCR-amplified and were made compatible for the construction of the BioBrick vectors. The *lacI* gene was amplified from the pREP4 plasmid with the GG_*lacI*_FW and GG_*lacI*_REW primers and a *de novo* strong RBS sequence (BBa_B0034, http://parts.igem.org/Part:BBa_B0034) was introduced (Indicated in red in Table S2). The DNA part containing the *glyA* gene was obtained from pQEαβRham using GG_*glyA*_FW and GG_*glyA*_REW primers comprised with a strong RBS sequence (BBa_J61100, http://parts.igem.org/Part:BBa_J61100), being this RBS sequence different to the *lacI* gene in order to avoid homologous recombination (Indicated in green in Table S2). The terminator sequence was maintained from the *glyA* native region, which was PCR-amplified from chromosomal *E. coli* K-12 [13]. *Bsa*I sites with two overhangs were introduced at the 5' and 3' prime in all assembled fragments to provide directional cloning and to prevent the religation of empty vector. All reaction DNA fragments were prepared equimolar to a concentration of 69 fmol·μL^{-1}. To each reaction, 0.5 μL of *Bsa*I and T4 ligase were added. Final reactions were incubated in a thermocycler as follows: 25-30 cycles (37 °C, 3 min; 16 °C, 4 min) and final step 50 °C, 5 min and 80 °C, 5 min. Thus, reactions were performed in one-step restriction-ligation (Figure 1A). Four BioBrick constructs were assembled, each one with a different constitutive promoter to tune the expression levels of *lacI* and *glyA* genes. The four vectors were named pSB1C3-J231XX, where the double X represents the last two digits of the promoter name (Figure 1B). Finally, 2-5 μL of assembly BioBrick constructs were transformed into 50 μL of *E. coli* DH5α.

pQE-FucA_puzzle (J23110)

The construction of vector derived from the pQE-FucA and the BioBrick vectors required a two-step assembly. The expression cassette J23110-*lacI-glyA* was amplified from pSB1C3-J23110-*lacI-glyA* using PJ/2_FW and PJ_REW primers. Both, PCR product and destination vector pQE-FucA were digested with *Bpu*10I and *Msc*I and subsequently extracted from agarose gel. The expression cassette was cloned into the pQE-FucA (double digested) obtaining the pQE-FucA_puzzle (J23110) (Figure S2A). Finally, the ligation product was transformed into the M15Δ*glyA* generating M15Δ*glyA* pQE-FucA_puzzle (J23110), from now on Puzzle strain.

pQE-FucA_puzzle (J23110)_AmpR$^-$

Vector pQE-FucA_puzzle (J23110) was double digested with *Eco*0109I and *Ahd*I in order to eliminate de *bla* gene. 5´-3´polymerase and 3´-5´exonuclease activities of DNA Polymerase I (Large) Klenow Fragment was used in order to end-removal and fill-in terminal unpaired nucleotides.

The blunting DNA reaction, composed of digested vector 0.5 mg, 1 µL of dNTPS 25 mM (Bioline) and 1 µL of DNA polymerase I (5U/µL) (NEB), was incubated at room temperature (RT) 20 min, followed by a heat inactivation step at 75 °C 10 min. The ligation reaction of the blunt ended DNA fragments and their respective plasmid backbones were carried out at 16 °C overnight using T4 ligase. The resulting ligation (Figure S2B) vector was transformed into *E. coli* M15Δ*glyA* and plated on defined medium (DM) plates, generating M15Δ*glyA* pQE-FucA_puzzle (J23110)_ AmpR$^-$, from now on AmpR$^-$ strain. Transformants were isolated and tested both, in DM and LB supplemented with ampicillin plates, as a positive and negative control, respectively. Selected transformants were able to grow in defined media but not in LB plates supplemented with ampicillin. Positive clones were validated as described previously.

Culture media

Luria Bertani (LB) medium, containing 10 g·L^{-1} peptone, 5 g·L^{-1} yeast extract and 10 g·L^{-1} NaCl, was used for pre-cultures.

Defined Medium (DM) used for shake flasks cultures contained per liter: 5 g glucose, 2.97 g K_2HPO_4, 0.60 g KH_2PO_4, 0.46 g NaCl, 0.75 g $(NH_4)_2SO_4$, 0.11 g $MgSO_4{\cdot}7H_2O$, 0.006 g $FeCl_3$, 0.025 g thiamine, 1.44 g $CaCl_2{\cdot}2H_2O$, 0.07 mL·100mL^{-1} medium of trace elements solution (TES) (TES contained per liter: 0.04 g $AlCl_3{\cdot}6H_2O$, 1.74 g $ZnSO_4{\cdot}7H_2O$, 0.16 g $CoCl_2{\cdot}6H_2O$, 2.18 g $CuSO_4{\cdot}5H_2O$, 0.01 g H_3BO_3, 1.42 g $MnCl_2{\cdot}6H_2O$, 0.01 g $NiCl_2{\cdot}6H_2O$, 0.23 g $Na_2MoO4{\cdot}5H_2O$). Agar plates prepared with DM contained 1.5% Agarose.

Stock solutions of kanamycin and chloramphenicol were prepared with a concentration of 100 mg·mL^{-1} and 30 mg·mL^{-1}, respectively, and stored at -20 °C. Ampicillin 100 mg·L^{-1} ethanol stock was prepared and stored at -20 °C. IPTG stock was prepared at 100 mM, and stored at -20 °C.

Vitamins, antibiotics, TES, $FeCl_3$, $MgSO_4{\cdot}4H_2O$, $CaCl_2{\cdot}2H_2O$ and inducer were sterilized by filtration (0.2 µm syringe filter made from a blend of cellulose esters, Sartorius). Glucose and saline solutions were separately sterilized by autoclaving at 121°C for 30 min.

Cultivation conditions

Pre-inoculum

Cryo-stocks stored at -80°C, were use to inoculate Falcon tubes with 15 mL of LB medium supplemented with the corresponding antibiotic if necessary. Growth was performed overnight at 37 °C with agitation.

Cultures

Three mL of overnight pre-inoculum were transferred into shake flasks containing 100 mL of DM, following the same growing conditions as pre-inoculum cultures. All cultivations were performed in a working volume of 100 mL in 500 mL volume-baffled shake flasks. To induce *fucA* expression, IPTG was added to a final concentration of 1 mM, when an OD_{600} of 1.5 was reached. The induction was maintained for 4 hours, sampling before induction and 1, 2 and 4 hours after induction.

Analytical methods

Cell concentration was determined by optical density (OD_{600}) measurements at 600 nm using a spectrophotometer (Uvicon 941 Plus, Kontrol). OD values were converted to biomass concentration expressed as Dry Cell Weight (DCW), being 1 OD_{600} equivalent to 0.3 gDCW·L^{-1} [18].

Glucose and acetate concentration were analysed in the broth. One milliliter of culture medium was separated from biomass by centrifugation at 14.000 rpm 6 min and filtered (0.45 μm membrane filter of cellulose esters, Millipore) prior to analysis. Glucose concentration was determined enzymatically on an YSI 2070 system (Yellow Spring System). Acetic acid was analyzed by HPLC (Hewlett Paackard 1050) equipped with an ICSep COREGEL 87H3 ICE-99-9861 (Transgenomic) column and IR detector (HP 1047), using 6 M H_2SO_4 (pH 2.0) as mobile phase, flow rate of 0.3 ml·min^{-1}, at 40 °C.

The biomass yield, $Y_{X/S}$ was calculated using the following equation:

$$Y_{X/S} = \frac{(DCW_{max} - DCW_0)}{(Glc_0 - Glc)} \qquad [1]$$

where, DCW_{max} and DCW_0 (g·L^{-1}) are the maximum and the initial biomass values, respectively. Glc_0 and Glc_f (g·L^{-1}) are initial and final value of glucose concentration, respectively.

The specific substrate uptake rate, q_S, is defined as follows:

$$q_S = \frac{\mu}{Y_{X/S}} \qquad [2]$$

where, q_S is given as grams of carbon per grams of biomass per hour (g·g^{-1}DCW·h^{-1}).

The maximum specific growth rate (μ_{max}) of the different strains is calculated by taking the natural log of the cell concentration and plotting it over time. The equation [3] shows the relationship between the cell concentration (X), maximum specific growth rate (μ_{max}) and time (t). Log-linearized Eq. [3] yields a linear relationship where the μ_{max} is represented by the slope of the linear portion in the plot of the natural log of cell concentration versus time.

$$X_t = X_0 \cdot e^{\mu_{max} \cdot t} \qquad [3]$$

$$lnX_t = lnX_0 + \mu_{max} \cdot t \quad [4]$$

where, X_t and X_0 are, in the linear zone, the OD600 or the cell concentration at any time (t) and at the beginning, respectively.

FucA quantification

Samples from culture broths were withdrawn, adjusted to a final OD_{600} of 3, centrifuged and then processed as previously described [19] and [13]. Briefly, pellets were resuspended in 100 mM TrisHcl (pH 7.5). Cell suspensions were placed in ice and sonicated over four pulses of 15 seconds each at 50W with 2 minutes intervals in ice between each pulse, using a Vibracell™ model VC50 (Sonics & Materials). Cellular debris were then removed by centrifugation and the cleared supernatant was collected for FucA analysis. One unit of FucA activity is defined as the amount of enzyme required to convert 1 µmol of fuculose-1-phosphate in DHAP and L-lactaldehyde for minute at 25 °C and pH 7.5 [13]. To quantify the amount of FucA relative to total intracellular soluble proteins, SDS-PAGE and Bradford protein assay were performed Average values were plotted with error bars. The error indicates the confidence interval with a confidential level of 90%.

Results

Comparison of FucA expression between M15[pREP4] and M15[pREP4] Δ*glyA* strain

Preliminary experiments between *E. coli* M15[pREP4] pQE-FucA and *E. coli* M15Δ*glyA*[pREP4] pQEαβFucA were performed in shake flask cultures in defined media (DM). Figures 2A and 2B compare biomass and FucA production profiles along time, for the M15[pREP4] and the M15Δ*glyA*[pREP4] strains, respectively. The reference M15[pREP4] strain presents a slightly higher maximum specific growth rate (μ_{max}) of 0.49 ± 0.01 h^{-1} compared to 0.44 ± 0.01 h^{-1} in M15Δ*glyA*[pREP4]. Moreover, Figures 2C and 2D present glucose consumption and acetate production profiles along time, for the M15[pREP4] and the M15Δ*glyA*[pREP4] strains, respectively. Substrate uptake rates (q_S) along the induction phase for both strains were calculated, being 0.37 ± 0.04 and 0.50 ± 0.13 $gGlc \cdot g^{-1}DCW \cdot h^{-1}$ for the M15[pREP4] and for the M15Δ*glyA*[pREP4], respectively. Moreover, whereas the M15[pREP4] strain reached a final production of 181 ± 5 $mgFucA \cdot g^{-1}DCW$, with an activity of 721 ± 82 $AU \cdot g^{-1}DCW$, these values were reduced to 67 ± 37 $mgFucA \cdot g^{-1}DCW$ 291 ± 24 $AU \cdot g^{-1}DCW$ respectively, in the M15Δ*glyA*[pREP4] strain (Figure 2A-B).
Figure 2E and 2F represented the SDS-PAGE for the M15[pREP4] and the M15Δ*glyA*[pREP4] strains, respectively. It can be clearly seen an increase in the SHMT band in the M15Δ*glyA*[pREP4] strain (Figure

2F). Moreover, in Table 1 it can be seen how the SHMT values of the M15Δ*glyA*[pREP4] strain, being around 90 mgSHMT·g^{-1}DCW, increased comparing with the M15[pREP4] strain, being around 20 mgSHMT·g^{-1}DCW.

pREP4 elimination

In order to obtain an expression system completely devoid of antibiotic resistance genes, we initially focused on the elimination of the pREP4 plasmid. The objectives were to i) obtain an expression system based on a single plasmid; ii) clone the *lacI* gene from the pREP4 plasmid to the pQE-expression vector. Accordingly, the pREP4 plasmid was eliminated from the M15Δ*glyA*[pREP4] pQEαβFucA system, obtaining the derived strain M15Δ*glyA*[A]. Shake flask cultures were performed in defined media supplemented with ampicillin (data not shown). An increase in the basal FucA production was expected, due to the removal of the repressor protein encoded by the *lacI* gene present on the pREP4 plasmid. Strikingly, no FucA production was detected in these cultures. To further understand this effect, the *lacI* gene from the pREP4 was amplified and cloned into the pQEαβFucA plasmid, obtaining the pQE-*lacI*-αβFucA expression vector. Then, shake flask cultures were performed with M15Δ*glyA*[B] strain harboring this plasmid (data not shown). However, FucA expression was not found. In order to ensure there was no loss of the expression vector from the cells, the plasmid segregational stability was carried out at different cultivation times before and after induction. The experiments confirmed that the M15Δ*glyA*[B] cells maintained the expression vector and, consequently, the lack of FucA expression was not the result of plasmid loss.
As mentioned in the Materials and Methods section, the T5 promoter has a double lac O region in order to guarantee a strong repression under non-induction conditions. The *lac* repressor, encoded by the *lacI* gene, binds very tightly to the promoter and ensures efficient repression of the strong T5 promoter interfering with the transcription of the gene of interest. In order to further understand whether the promoter leakiness in the absence of repressor is due to structural instability, T5 promoter region isolated from several non-producing M15Δ*glyA*[A] constructs was sequenced. Interestingly, a deletion in the *lac* O regions was observed, probably due to recombination events in the homology region (data not shown).

Tuning of *lacI* and *glyA* expression levels

A series of FucA expressing strains presenting 4 different constitutive transcriptional levels of *lacI* were constructed. Moreover, because the first generation of *glyA*-based auxotrophic system contained the *glyA* gene under the control of the P3 constitutive promoter, resulting in relatively high amounts of its product, the same set of 4 promoters were tested to reduce the transcriptional levels of the *glyA* gene (Vidal L et al., 2008). Thus, an expression cassette was settled where the *lacI* and *glyA* genes were cloned under the control of the four constitutive promoters.

The aim was to find the suitable promoter with strength enough to synthetize the minimum amount of LacI inhibitor molecules preventing "promoter leakiness", as well as the minimal *glyA* transcriptional levels required to maintain plasmid-bearing cells and optimal cell growth in defined media.

The four resulting expression vectors were co-transformed with the pQE-FucA plasmid into M15Δ*glyA*. The four expression systems generated were named M15Δ*glyA*[C00], [C11], [C10] and [C17] strains. Biomass and enzyme production were analyzed along time and are presented in Figure 3A, 3B, 3C and 3D, for M15Δ*glyA*[C00], M15Δ*glyA*[C11], M15Δ*glyA*[C10] and M15Δ*glyA*[C17] strains, respectively.

The μ_{max} measured in the different cultures for the M15Δ*glyA*[C11], M15Δ*glyA*[C10] and M15Δ*glyA*[C17] transformants were 0.37 ± 0.01 h^{-1}, 0.48 ± 0.01 h^{-1} and 0.41 ± 0.01 h^{-1}, respectively (Table 2). M15Δ*glyA*[C00] strain presented significantly higher μ_{max} being 0.62 ± 0.05 h^{-1} (Table 2). The over-expression of FucA for the 4 selected transformants. The M15Δ*glyA*[C10] results the strain with the higher production both in term of mass and activity, being 83 ± 7 mg·g^{-1}DCW and 574 ± 49 AU·g^{-1}DCW, respectively (Figure 3C).

On the other hand, Table 2 and Figure 4 presents the glucose consumption and acetate production profiles along time for the 4 strains: 4A, 4B, 4C and 4D, for M15Δ*glyA*[C00], M15Δ*glyA*[C11], M15Δ*glyA*[C10] and M15Δ*glyA*[C17], respectively. It can be observed that the higher q_S value, being 0.79 ± 0.10 g·g^{-1}DCW·h^{-1}, belongs to the M15Δ*glyA*[C00] strain, with an acetate production of 1.50 ± 0.10 gAc·g^{-1}DCW. While the M15Δ*glyA*[C10] strain presents the lower acetate amount of 0.70 ± 0.12 gAc·g^{-1}DCW.

Expression vector optimization

The M15Δ*glyA*[C11] was previously selected as the strain with highest FucA production and specific activity among the 4 different constructs. To further optimize the expression system, the next goal was the construction of a single vector harboring both the *fucA* gene under control of the inducible T5 promoter and the *lacI-glyA* cassette cloned under the J23110 constitutive promoter. Such plasmid was constructed as described in Materials and Methods section and transformed into M15Δ*glyA*, yielding *E. coli* M15Δ*glyA* pQE-FucA_puzzle (J23110) from now on Puzzle strain.

The μ_{max} of the Puzzle strain was 0.45 ± 0.01 h^{-1}. Maximum FucA mass and FucA specific activity reached were 162 ± 7 mg^{-1} FucA·g^{-1}DCW and 984 ± 35 AU·g^{-1}DCW, respectively (Figure 5A). Besides, the amount of acetate production for the Puzzle strain results 0.42 ± 0.03 g·g^{-1}DCW.

Furthermore, in Table 1, SHMT values are presented, being 66 ± 17, 62 ± 10, 54 ± 14 and 54 ± 7 mgSHMT·g^{-1}DCW, for the PI, 1h, 2h and 4h induction samples, respectively.

Development of an antibiotic-free plasmid maintenance

Lastly, an expression system completely devoid of antibiotic resistance genes was constructed by removing the *bla* gene from the expression vector (Figure S2B). The corresponding strain was named *E. coli* M15Δ*glyA* pQE-FucA_puzzle (J23110)_AmpR-, so-called AmpR- strain.

Shake flasks cultures were performed in fined medium (DM) without any antibiotic supplementation. The time-profiles of the biomass, glucose consumption, acetate and FucA (mass and specific activity) were analyzed. Results are presented in Figure 6. The μ_{max} was calculated being μ_{max} 0.41 ± 0.01 h^{-1}. In terms of FucA production, the point of maximum activity corresponds to 1309 ± 42 AU·g^{-1}DCW with a production in mass of 219 ± 5 mgFucA·g^{-1}DCW after 4 h of induction (Figure 6A). Finally, SHMT values were calculated for each time point of induction. The results are presented in Table 1, where the pre induction sample with 53 ± 1 mgSHMT·g^{-1}DCW represents the sample with the higher amount of SHMT.

Discussion

In the present work, we have further developed a novel expression system based on an antibiotic-free plasmid maintenance mechanism. Our stepwise design approach resulted in increased production levels, up to 3.8-fold in terms of FucA yield (mg·g^{-1}DCW) and 4.5-fold in terms of FucA activity (AU·g^{-1}DCW), compared to the reference M15[pREP4] expression system.

The comparison between the reference M15[pREP4] and the M15Δ*glyA*[pREP4] *E. coli* strains demonstrated that the later presents slightly lower specific growth rate, decreasing from 0.49 ± 0.01 h^{-1}, of the reference strain, to 0.44 ± 0.01 h^{-1} of the M15Δ*glyA*[pREP4] strain. This effect may be caused by the increase in the metabolic burden due to the maintenance of the expression vector in the M15Δ*glyA* strain. The presence of the *glyA* gene in the vector results in a higher load of this gene due to the multiple copies of the plasmid. Furthermore, by comparing the qS values of both strains, it can be clearly seen that the M15Δ*glyA*[pREP4] strain showed an increase in the specific glucose uptake rate from 0.37 ± 0.04 to 0.50 ± 0.13 gGlc·g^{-1}DCW·h^{-1}. As a consequence, the M15Δ*glyA*[pREP4] strain accumulated higher amounts of acetate throughout, reaching a final concentration of 0.54 ± 0.03 g·L^{-1} as it can be observed in Figure 2D This results in higher acetate specific production rates. This is coherent with previous studies on acetate under aerobic conditions, pointing at the unbalance between glycolysis rates and the TCA-cycle limited capacity of *E. coli* [20]. Furthermore, it has been reported that the recombinant protein production is significantly reduced by acetate accumulation [21]. Such effect can be observed in this study, were both FucA activity (AU·g^{-1} DCW) and FucA mass (mg·g^{-1}DCW) (Figure 2B) decrease more than 50 % when comparing the M15Δ*glyA*[pREP4] strain to the reference M15[pREP4] (Figure 2A).

Noteworthy, the metabolic burden is caused not only due to the overexpression of the protein of interest but also to the expression of other plasmid-encoded genes, that is, the *glyA* overexpression may also

contribute [22]. In fact, the *glyA* gene encoded in the high-copy plasmid leads to substantially higher amounts of its product (SHMT) accumulated as soluble protein in the cytoplasm, compared to the reference strain containing a single copy of *glyA* in the genome as observed in SDS-PAGE (Figure 2E-F) and in Table 1. The SHMT production (mgSHMT·g^{-1}DCW) increased more than 4.5-fold when moving from the M15[pREP4] to the M15Δ*glyA*[pREP4] strain. This observation suggested that *glyA* overexpression imposed a significant burden to the metabolism of the host cell, thereby affecting negatively FucA expression levels and the μ_{max}. These preliminary results suggested the regulation of the *glyA* expression levels as an important parameter to be taken into account for further improvement of the expression system and optimization of protein yields.

Interestingly, in this work we report the lack of FucA expression in the system with no *lacI* gene. This effect may be related to T5 promoter leakiness in absence of LacI repressor protein, leading to plasmid structural instability due to recombination events, as supported by the sequencing data. Alternatively, a possible explanation for the lack of FucA expression in the single plasmid system may be that the copy number of the *lacI* gene increases when cloned into the pQE-40 vector, resulting in significantly higher levels of intracellular LacI. In fact, the pQE vector is based on the plasmid replication origin ColE1, which presents a copy number 2-fold higher compared with the P15A replicon of pREP4 [1].

These experiments confirmed that *glyA* and *lacI* co-expression were required. Fine-tuning the co-expression of the two genes allowed to i) reduce the metabolic burden related to plasmid-encoded proteins and, ii) optimize the regulation and induction of the foreign gene expression, when engineering parts of the reference two-plasmid system into a single plasmid.

For this reason, four different expression cassettes were constructed where the *lacI* and *glyA* genes were placed under the control of a set of four constitutive promoters, covering a wide range of transcriptional efficiencies. The resulting selected transformants were named M15Δ*glyA*[C00], [C11], [C10] and [C17] and tested in triplicate. The specific growth rate measured in the different cultures showed a similar behavior for all the transformants except for those with the J23100 promoter. The strongest promoter, which is the one that presented a μ_{max} 1.3 fold higher than the reference strain M15[pREP4] and the other three strains (Table 2). Conversely, the use of a stronger constitutive promoter for the *lacI* and *glyA* expression such as the J23100 resulted in low detection levels of FucA, both, in terms of mass and activity.

Therefore, a higher growth rate could be explained as follows: higher constitutive *lacI* expression level may lead to a reduction of the *fucA* expression and subsequently decrease the metabolic burden. Furthermore, when comparing q_S values during the induction phase, it can be clearly seen that the M15Δ*glyA*[C00] strain shows higher glucose specific uptake rate, being 0.79 ± 0.10 g·g^{-1}DCW·h^{-1} (Table 2). Consistently, this strain resulted in the production of the highest yields of acetate, reaching 1.50 ± 0.10 gAc·g^{-1}DCW.

According to the results shown in Figure 3C and Table 2, the selected expression cassette for *lacI* and *glyA*, was the one where both genes were placed under the control of the constitutive promoter J23110. This promoter, which is in the lower range of the tested *lacI* and *glyA* transcriptional levels, seems to down-regulate their transcriptional levels. This suggested that the reduced expression of *lacI* and *glyA*

expression seems to have reduced the energy demand and the building blocks necessary for *glyA* synthesis. In addition, T5 promoter leakiness was minimized, resulting in an overall reduced metabolic burden. This result is reflected in the fact that the μ_{max} of this strain was comparable to that of the M15[pREP4] reference strain (0.48 ± 0.01 h^{-1}) under pre-induction conditions. Furthermore, the M15Δ*glyA*[C10] strain presented the lowest q_S (0.44 ± 0.06 $g{\cdot}g^{-1}DCW{\cdot}h^{-1}$) and acetate yields (0.70 ± 0.12 $g{\cdot}g^{-1}DCW$) compared to the other three constructs and the highest FucA production, both in terms of mass, 83 ± 7 $mg{\cdot}g^{-1}DCW$ and activity, 574 ± 49 $AU{\cdot}g^{-1}DCW$.

Interestingly, FucA production values increased even more in the strain Puzzle strain compared to the previous system with two plasmids. In particular, while the μ_{max}, being 0.45 ± 0.01 h^{-1}, was still comparable to those from the preceding 2-plasmid construct and original reference strains (being 0.48 ± 0.01 h^{-1} and 0.49 ± 0.01 h^{-1}, respectively), the maximum FucA mass and FucA specific activity reached were 162 ± 7 mg^{-1} $FucA{\cdot}g^{-1}DCW$ and 984 ± 35 $AU{\cdot}g^{-1}DCW$, respectively (Figure 5A and Table 3). Comparing these values with those obtained with the M15[pREP4] reference strain (181 ± 5 mg $FucA{\cdot}g^{-1}$ DCW and 721 ± 82 $AU{\cdot}g^{-1}$ DCW), it can be observed how the specific activity increased 1.4-fold even though the amount of the recombinant protein was relatively lower. Besides, the Puzzle strain presented a reduction in the amount of acetate production, being 0.42 ± 0.03 $g{\cdot}g^{-1}DCW$, in comparison with the 0.73 ± 0.04 $g{\cdot}g^{-1}DCW$ of the reference strain.

These results suggest that transcriptional tuning of *lacI* expression levels is a key factor to improve *fucA* expression regulation, leading to a higher FucA specific activity. Moreover, the tuning of *glyA* levels has a positive effect on the reduction of the metabolic load due to expression of plasmid-encoded genes (also reflected in the reduced acetate production). In fact, SHMT values were almost 50% reduced comparing the Puzzle with the M15Δ*glyA*[pREP4] strains (Table 1). These results are in accordance with the observation by Mairhofer et al. [23], who demonstrated that the folding machinery is severely overstrained in the plasmid-based expression system compared with the plasmid-free cells due to the different transcriptional profiles.

Finally, the complete deletion of the antibiotic resistance gene has been achieved resulting in the $AmpR^-$ strain. A slightly decrease in the μ_{max}, a value of 0.41 ± 0.01 h^{-1} was observed compared to the M15[pREP4] and Puzzle strains, which showed a μ_{max} of 0.49 ± 0.02 h^{-1} and 0.45 ± 0.01 h^{-1}, respectively (Figure 6 and Table 3). This strain presented a significant increase both for the FucA specific mass and FucA specific activity. After 4 h of induction it was found maximum activity that corresponded to 1309 ± 42 $AU{\cdot}g^{-1}DCW$ and 219 ± 5 $mgFucA{\cdot}g^{-1}DCW$.

Comparing with all the previous constructs, FucA over-production using the antibiotic free-plasmid system is higher than any previous developed system studied. In particular, as it can be seen in Table 3 and in Figure 7, FucA yields in the $AmpR^-$ strain is: i) more than 1.2-fold higher comparing with the M15[pREP4] and the Puzzle strains; ii) 2.6-fold higher comparing with the M15Δ*glyA*[C10] strain and iii) three- fold higher referred to the M15Δ*glyA*[pREP4] strain. Noteworthy, the FucA activity, in terms of $AU{\cdot}g^{-1}DCW$, increased through the different stepwise improvements performed along this work. The best performing

engineered strain reached 4.5-fold higher values compared to the first M15Δ*glyA*[pREP4] strain. Additionally, the acetate production, expressed as $gAc \cdot gDCW^{-1}$, was also significantly reduced.

Conclusions

In this work we have applied rapid assembly strategies for the construction of improved expression systems that are useful for recombinant protein production. Using as a reference expression system commercially available, we have obtained an improved system that resulted in higher protein yields and devoid of antibiotic supply.

This case-study demonstrates that tuning the expression levels of *lacI* and *glyA* genes, which encode for the lac repressor and the auxotrophic selection marker protein, respectively, results into a reduction of the metabolic burden leading to a better stability of expression system. This fact allows an improvement of the recombinant protein production due to the alleviation of the metabolic burden and a reduction of acetate secretion. The main advantage of this engineered expression system devoid of antibiotic resistance markers is that it can be used as a platform for the production of a wide range of heterologous proteins where the use of antibiotics is restricted. Our work allows versatile and tuneable levels of expressed proteins at will, and we envisage that it can be potentially used in a wide range of applications and biotechnological processes with a significant reduction of production time and upstream costs.

Nonetheless, to broaden the understanding and commercial exploitation using this novel expression platform, studies at bioreactor scale using biomass concentrations comparable to industrial processes should be done.

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Ethical statement/conflict of interest

All authors concur with the submission and agree with its publication. The authors declare that they have no conflict of interest.

The authors confirm that this work is original and has not been published elsewhere nor is it currently under consideration for publication elsewhere.

The manuscript does not contain experiments using animals or human studies.

Authors' contributions

MP: Performed all experiments, acquisition and analysis of all the data, as well as in drafting of the manuscript. AFC: Contributed to the conceptual design of the study and manuscript editing. AJ: Involved in the design of constructs and manuscript edition. CdM, GC and PF: Contributed to the overall conceptual design of the study and data interpretation, as well as in drafting and revision of the manuscript. All Authors read and approved the manuscript.

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Figure Legends

Fig. 1 Golden Gate Assembly Method. **A**) Schematic diagram of Golden Gate assembly method to facilitate the construction of the new BioBrick vectors. **C**) Representation of the four pSB-J231XX vectors, each one with one of the four constitutive promoters. J231XX, constitutive promoter where the double X represents the last two digits of the promoter name (J23100, J23111, J23110 and J23117); RBS, ribosome binding site (purple for the *lacI* gene and pink for the *glyA* gene); lacI, *lacI* gene; glyA, *glyA* gene; term, termination sequence for the *glyA* gene; *camR*, chloramphenicol resistance gene; pMB1, replication origin.

Fig. 2 A, B, C and **D** represent the profiles along time of shake flasks cultures performed per triplicate in DM media at 37°C with agitation. (○) Biomass DCW ($g \cdot L^{-1}$),(▲) enzyme activity ($AU \cdot gDCW^{-1}$), (■) specific mass production content ($mgFucA \cdot gDCW^{-1}$), (▼) Glucose ($g \cdot L^{-1}$) and (●) Acetic Acid ($g \cdot L^{-1}$). The arrow indicates the IPTG pulse for the induction. **E** and **F** represent the SDS-PAGE of shake flasks culture's samples, where Lane M: molecular weight marker. 1, 2, 3 correspond to the shake flask culture replicates while the PI (pre induction) and 1 h, 2h and 4h correspond to the time after induction. The 26 kDa FucA and 46 kDa SHMT bands are indicated in the Figure. **A**, **C** and **E** refer to the M15[pREP4] strain while **B**, **D** and **F** refer to the M15Δ*glyA*[pREP4] strain.

Fig. 3. (○) Biomass DCW($g \cdot L^{-1}$), (▲) enzyme activity ($AU \cdot g^{-1}DCW$) and (■) specific mass production ($mgFucA \cdot g^{-1}DCW$) profiles along time in defined media at 37°C with agitation of the 4 different transformants: **A**) M15Δ*glyA*[00] **B**) M15Δ*glyA*[11] **C**) M15Δ*glyA*[10] **D**) M15Δ*glyA*[17]. The arrow indicates the IPTG pulse for the induction.

Fig. 4 (▼) Glucose ($g \cdot L^{-1}$) and (●) Acetic Acid ($g \cdot L^{-1}$) profiles along time in defined media shake flasks cultures performed per triplicate in DM media at 37°C with agitation of the 4 different transformants: **A**) M15Δ*glyA*[00] **B**) M15Δ*glyA*[11] **C**) M15Δ*glyA*[10] **D**) M15Δ*glyA*[17]. The arrow indicates the IPTG pulse for the induction.

Fig. 5 A) (○) Biomass DCW ($g \cdot L^{-1}$), (▲) enzyme activity ($AU \cdot g^{-1}DCW$), (■) specific mass content ($mgFucA \cdot g^{-1}DCW$) and **B**) (▼) Glucose ($g \cdot L^{-1}$) and (●) Acetic Acid ($g \cdot L^{-1}$) profiles, along time in a defined medium shake flasks cultures performed at 37°C for the Puzzle strain. The arrow indicates the 1mM IPTG pulse for the induction.

Fig. 6 A) (○) Biomass DCW($g \cdot L^{-1}$), (▲) enzyme activity ($AU \cdot g^{-1}DCW$), (■)specific mass ($mgFucA \cdot g^{-1}DCW$) and **B**) (▼) Glucose ($g \cdot L^{-1}$) and (●) Acetic Acid ($g \cdot L^{-1}$) profiles, along time in a defined medium shake flasks cultures performed at 37°C 150 rpm for the AmpR- strain. The arrow indicates the IPTG pulse for the induction.

Fig. 7 A) Maximum enzyme activity ($AU \cdot g^{-1}DCW$) and **B)** Maximum specific mass ($mgFucA \cdot g^{-1}DCW$) along the induction phase for the principal strains presented along this study. A dash-dot line indicates the value of the M15[pREP4] reference strain.

Table 1 SHMT production (mg·g^{-1}DCW) along the induction phase for the principal strains presented along this study. PI (pre induction) and 1 h, 2h and 4h correspond to the time after induction.

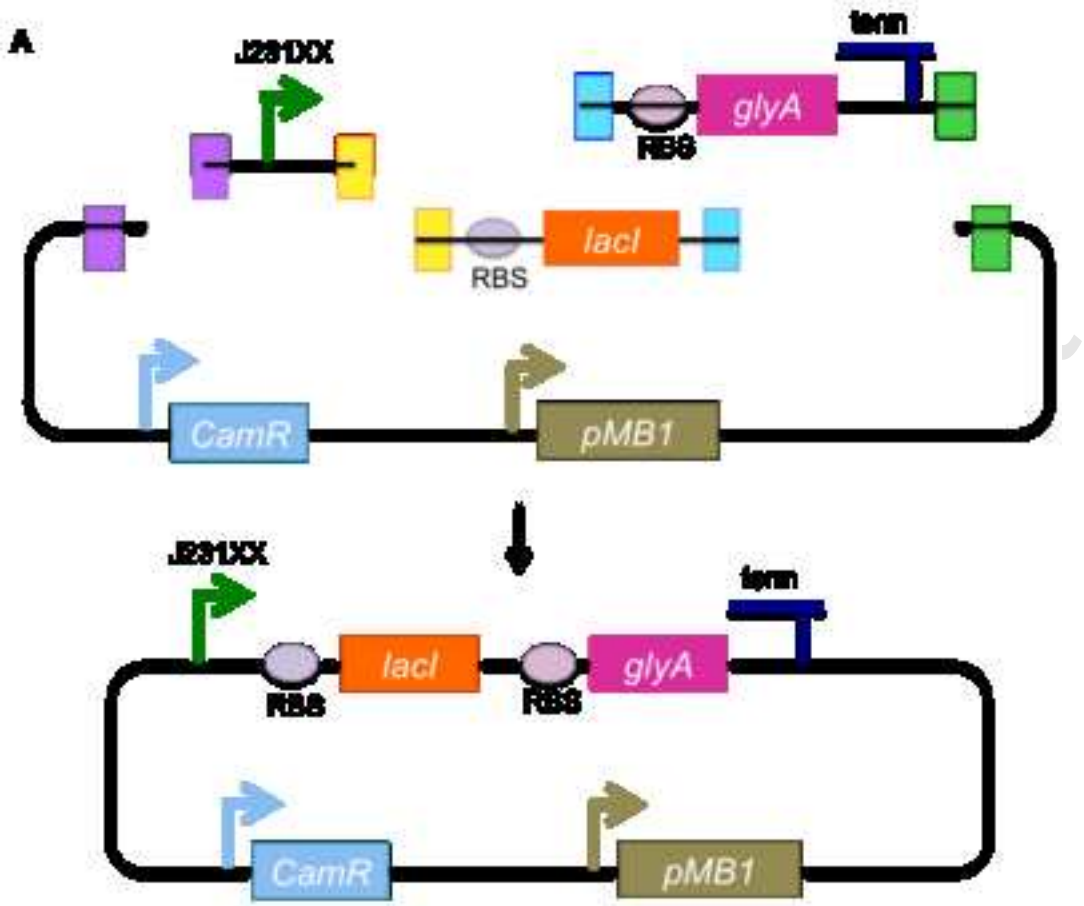
	M15[pREP4]	M15Δ*glyA*[pREP4]	Puzzle	AmpR-
PI	27 ± 14	92 ± 14	66 ± 17	53 ± 1
1h	14 ± 1	95 ± 7	62 ± 10	44 ± 11
2h	12 ± 4	91 ± 11	54 ± 14	48 ± 6
4h	13 ± 1	90 ± 14	54 ± 7	50 ± 10

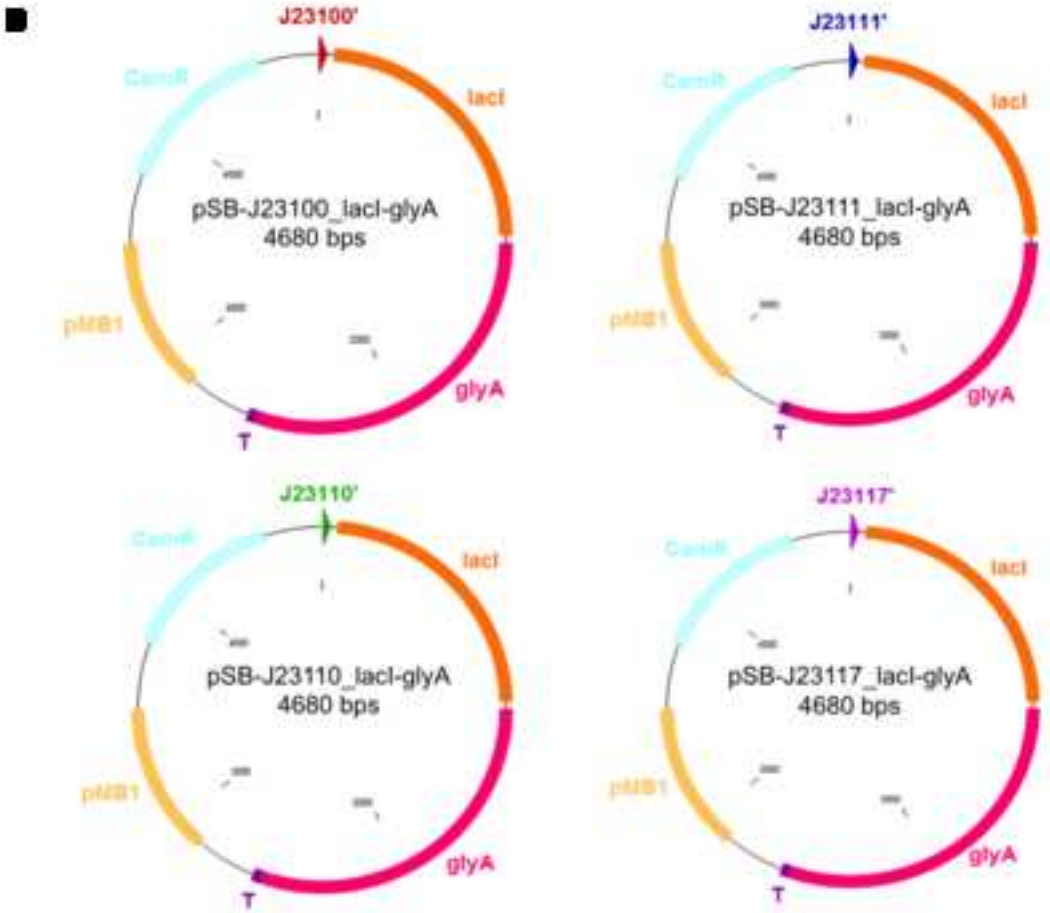
Table 2 Maximum specific growth rate (μ_{max} h^{-1}), FucA activity (AU·g^{-1}DCW), FucA mass (mg·g^{-1}DCW), q_S of the induction phase and the maximum acetate yield (g·g^{-1}DCW) for each of the fours selected transformants M15Δ*glyA*[C00], M15Δ*glyA*[C11], M15Δ*glyA*[C10] and M15Δ*glyA*[C17]. The values represent the sample after 2 hour of induction.

Transformant	μ_{max} (h^{-1})	FucA Activity (AU·g^{-1}DCW)	FucA mass (mg·g^{-1}DCW)	q_S (g·g^{-1}DCW·h^{-1})	Acetate yield (g·g^{-1}DCW)
M15Δ*glyA*[C00]	0.62 ± 0.05	131 ± 35	22 ± 9	0.79 ± 0.10	1.50 ± 0.10
M15Δ*glyA*[C11]	0.37 ± 0.01	233 ± 7	66 ± 4	0.42 ± 0.09	1.12 ± 0.09
M15Δ*glyA*[C10]	0.48 ± 0.01	574 ± 49	83 ± 7	0.44 ± 0.06	0.70 ± 0.12
M15Δ*glyA*[C17]	0.41 ± 0.01	194 ± 24	50 ± 5	0.58 ± 0.01	1.11 ± 0.05

Table 3 Maximum FucA activity (AU·g^{-1}DCW), maximum FucA mass (mg·g^{-1}DCW), μ_{max} and maximum acetate yield (g·g^{-1}DCW) along the induction phase for the principal strains presented along this study.

E. coli strains	μ_{max} (h^{-1})	FucA activity (AU·g^{-1}DCW)	FucA mass (mg·g^{-1}DCW)	Acetate yield (g·g^{-1}DCW)
M15[pREP4]	0.49 ± 0.02	721 ± 82	181 ± 5	0.73 ± 0.04
M15Δ*glyA*[pREP4]	0.44 ± 0.01	291 ± 24	67 ± 37	0.90 ± 0.04
M15Δ*glyA* [C10]	0.48 ± 0.02	574 ± 49	83 ± 7	0.70 ± 0.12
Puzzle	0.45 ± 0.01	984 ± 35	162 ± 7	0.42 ± 0.03
AmpR$^-$	0.41 ± 0.01	1309 ± 42	219 ± 5	0.37 ± 0.01





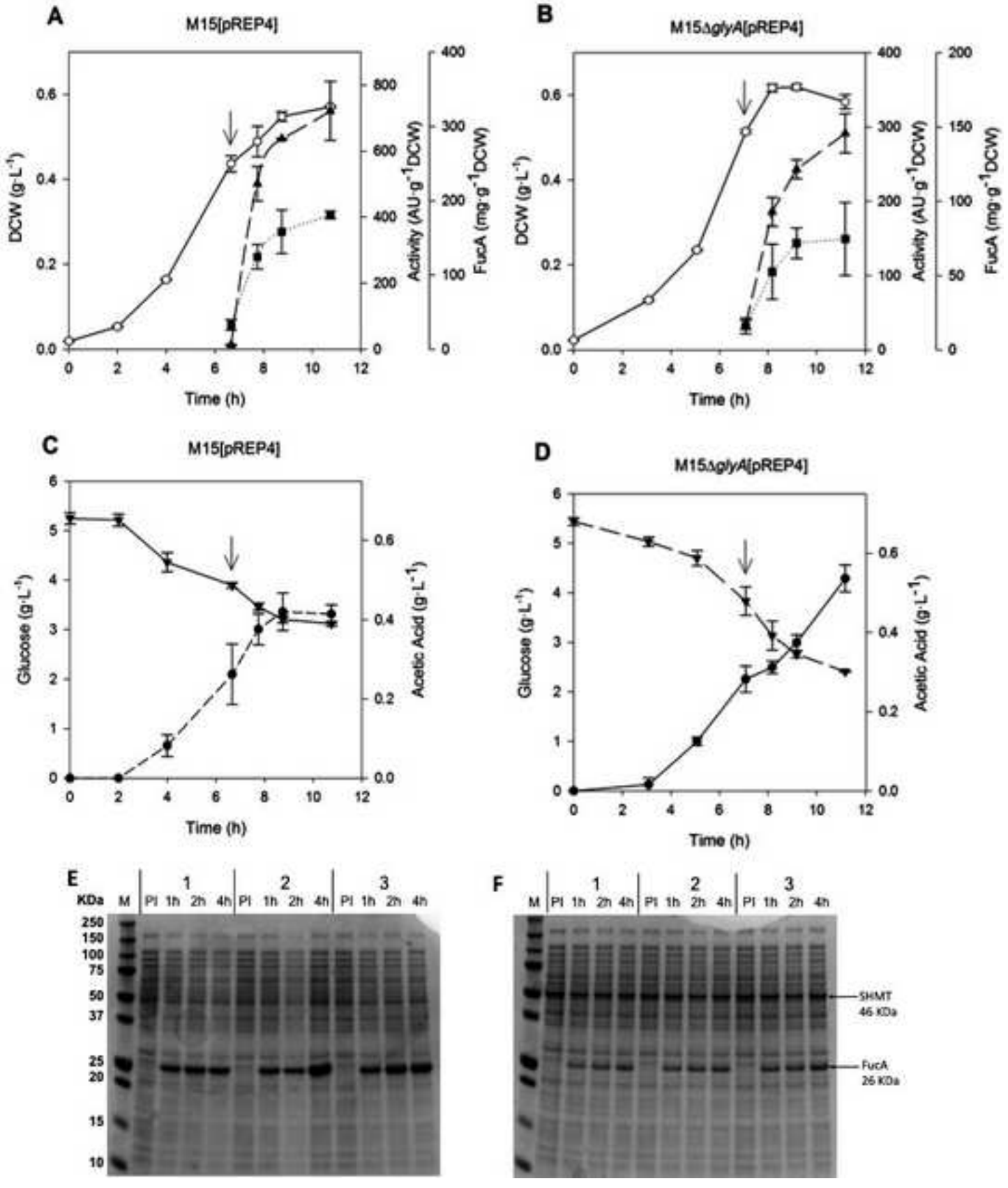


Figure 3

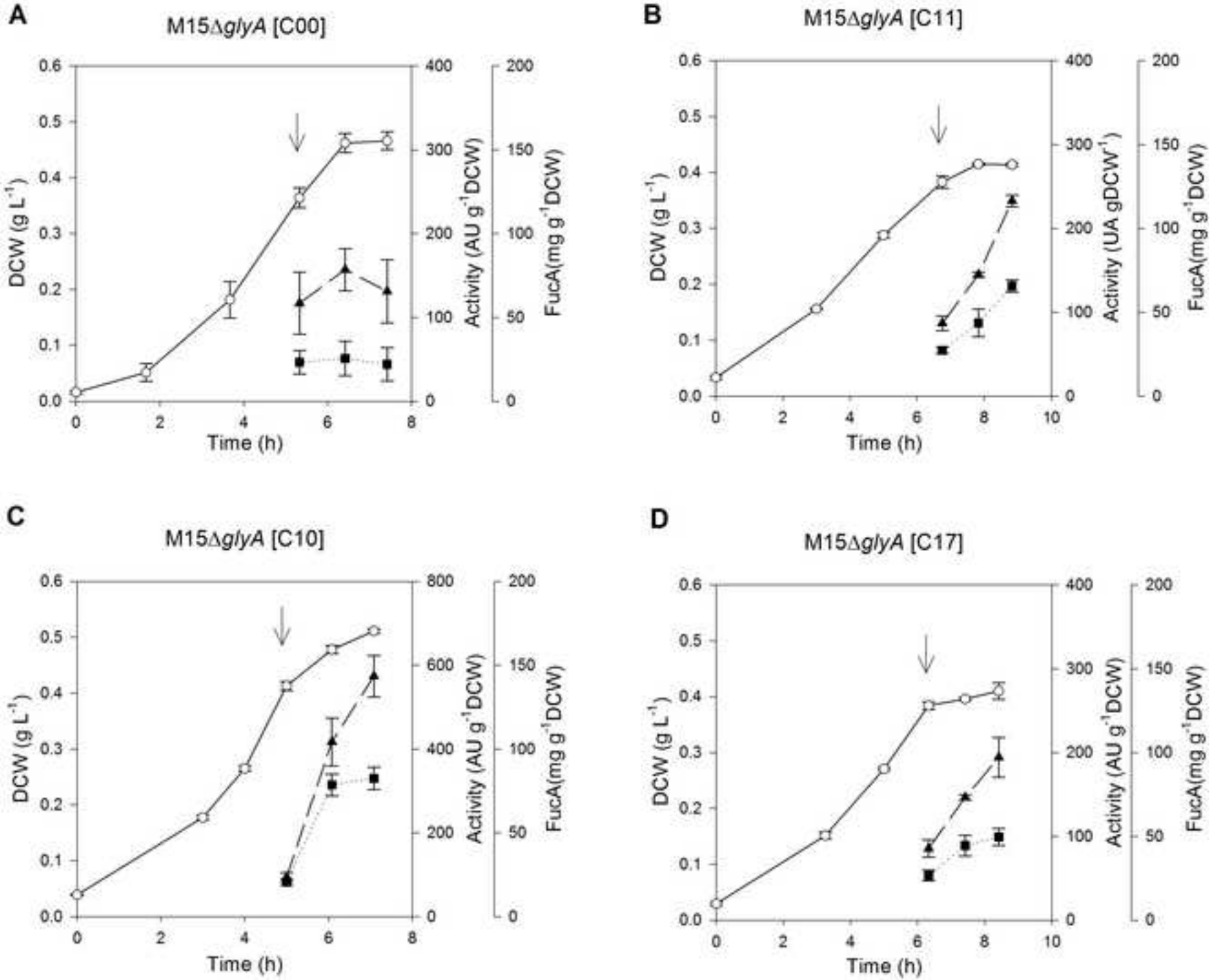

A
M15ΔglyA [C00]
B
M15ΔglyA [C11]
C
M15ΔglyA [C10]
D
M15ΔglyA [C17]
DCW (g L-1)
Activity (AU g-1DCW)
Activity (UA gDCW-1)
FucA(mg g-1DCW)
Time (h)

Figure 4

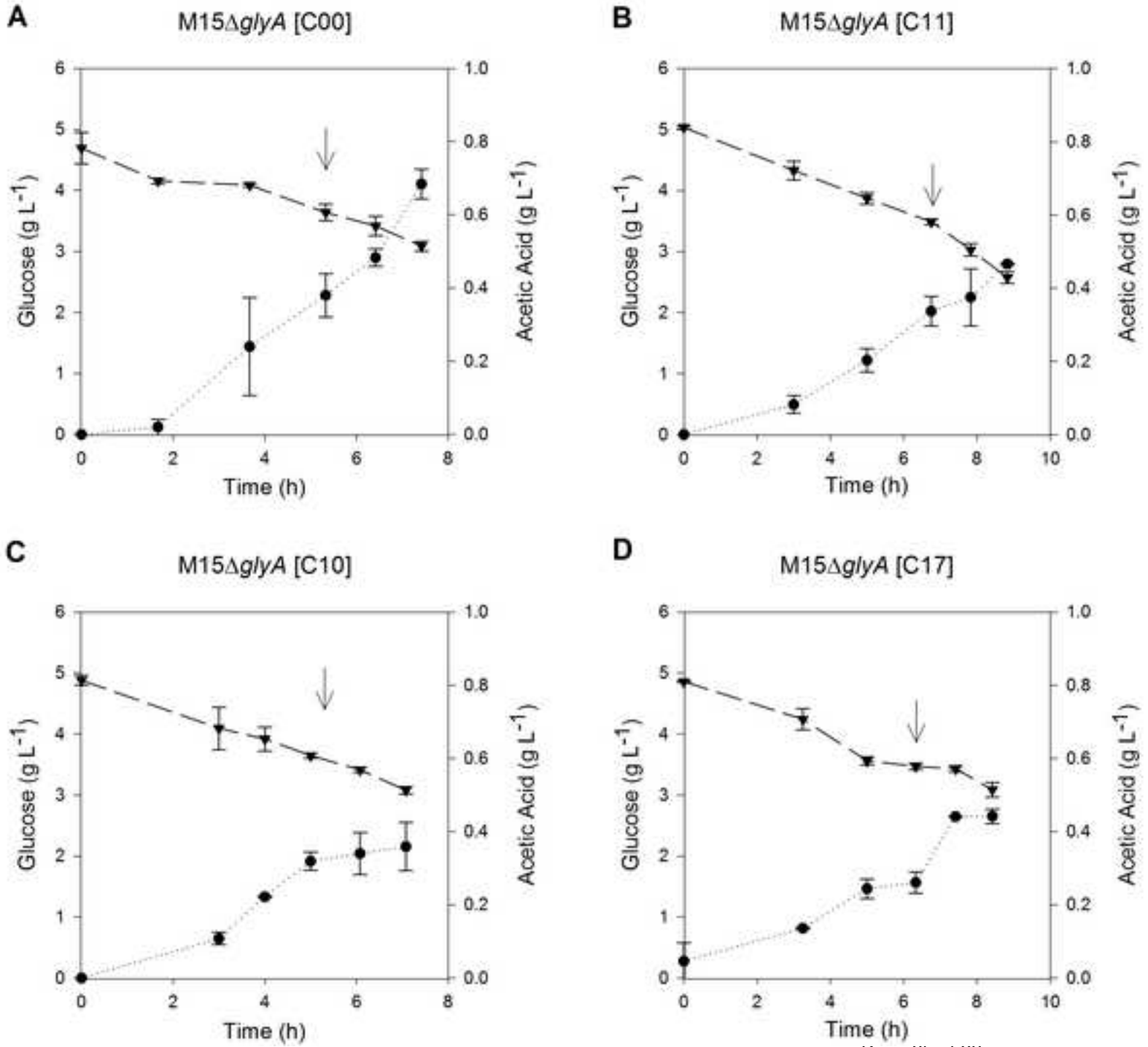

A
M15ΔglyA [C00]
B
M15ΔglyA [C11]
C
M15ΔglyA [C10]
D
M15ΔglyA [C17]
Glucose (g L-1)
Acetic Acid (g L-1)
Time (h)

Figure 5

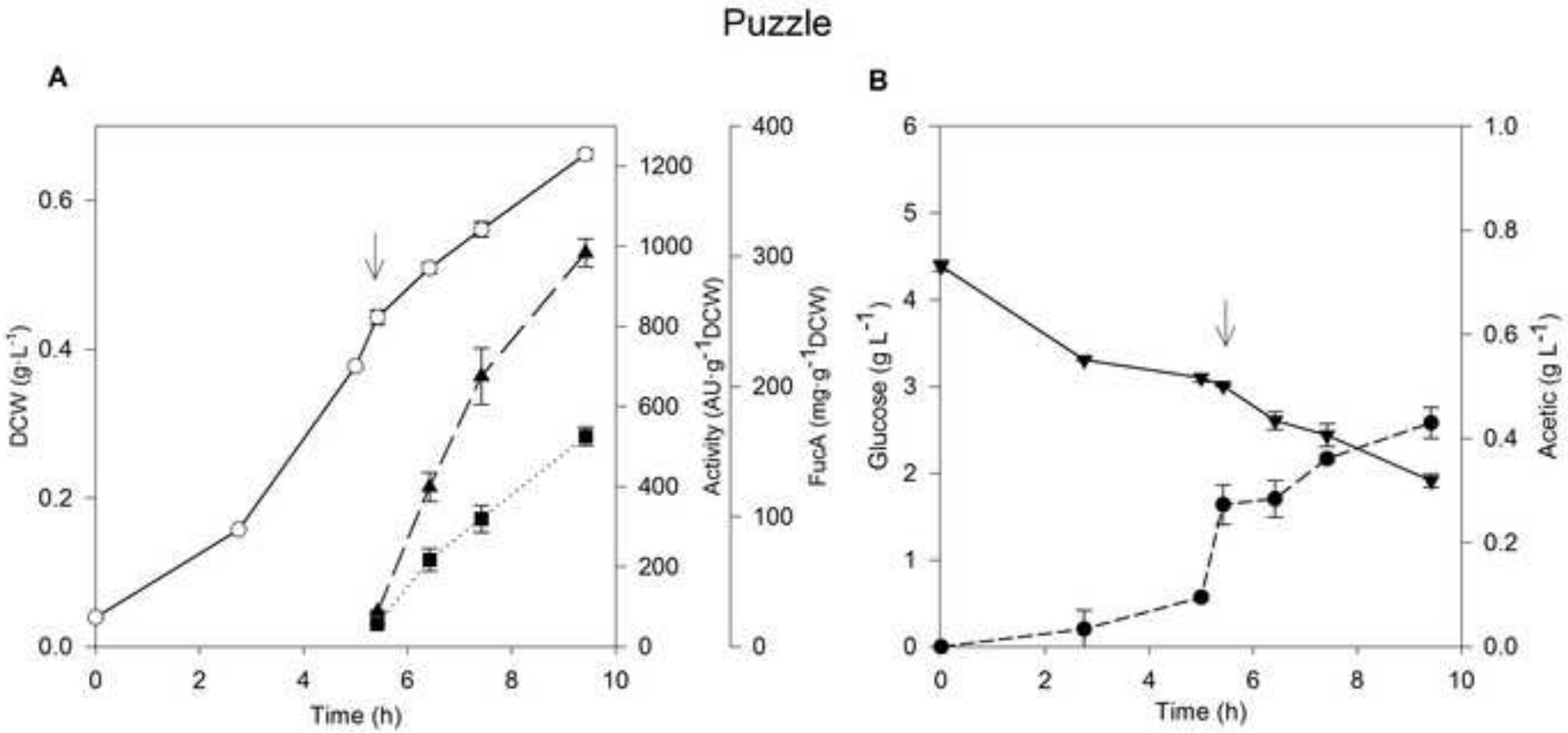
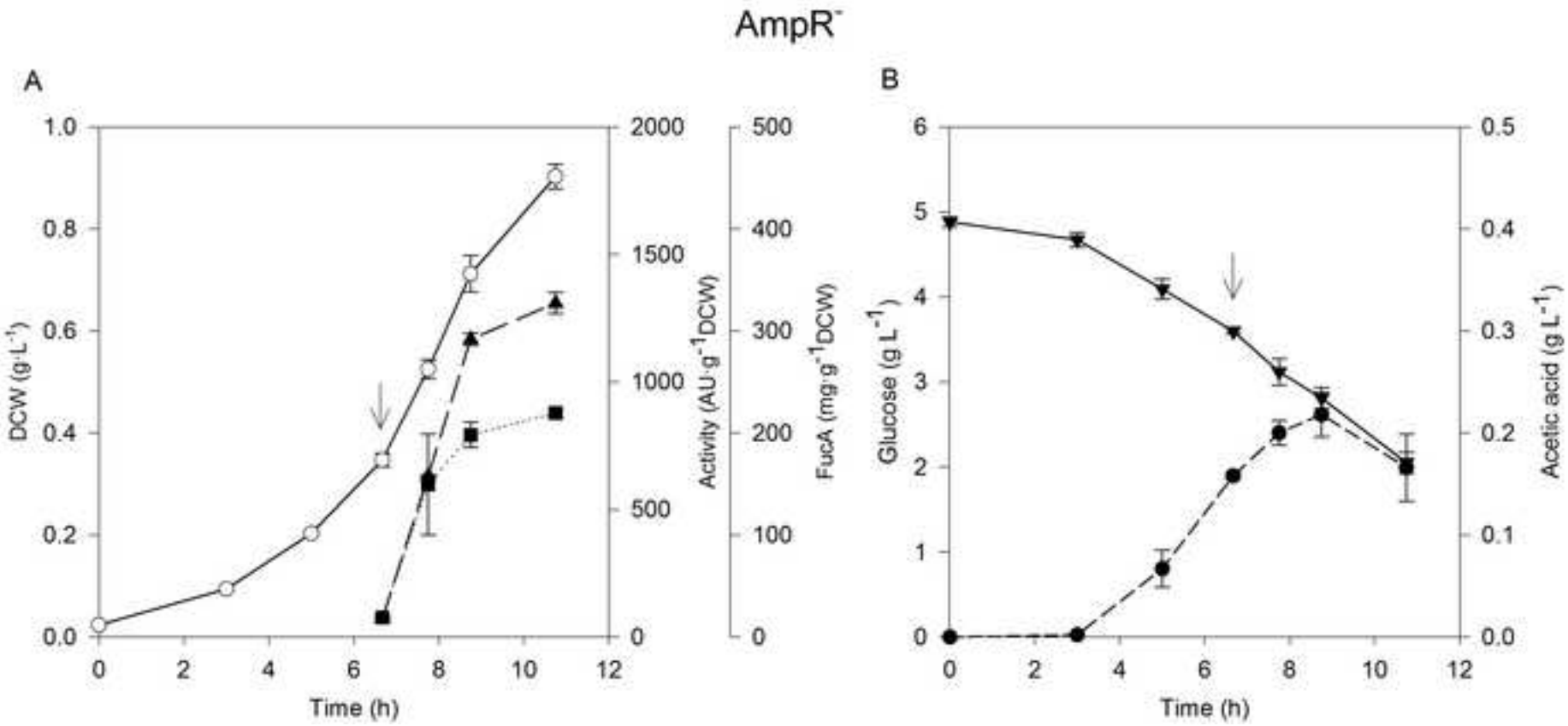

Puzzle
A
B
DCW (g·L-1)
Activity (AU·g-1DCW)
FucA (mg·g-1DCW)
Time (h)
Glucose (g L-1)
Acetic (g L-1)
Time (h)

Figure 6


AmpR-
A
B
DCW (g·L-1)
Activity (AU·g-1DCW)
FucA (mg·g-1DCW)
Time (h)
Glucose (g L-1)
Acetic acid (g L-1)
Time (h)