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De novo creation of MG1655-derived *E. coli* strains specifically designed for plasmid DNA production

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Abstract

The interest in plasmid DNA (pDNA) as a biopharmaceutical has been increasing over the last several years, especially after the approval of the first DNA vaccines. New pDNA production strains have been created by rationally mutating genes selected on the basis of *E. coli* central metabolism and plasmid properties. Nevertheless, the highly mutagenized genetic background of the strains used makes it difficult to ascertain the exact impact of those mutations. To explore the effect of strain genetic background, we investigated single and double knockouts of two genes, *pykF* and *pykA*, which were known to enhance pDNA synthesis in two different *E. coli* strains, MG1655 (wild-type genetic background) and DH5α (highly mutagenized genetic background). The knockouts were only effective in the wild-type strain MG1655, demonstrating the relevance of strain genetic background and the importance of designing new strains specifically for pDNA production. Based on the obtained results, we created a new pDNA production strain starting from MG1655, by knocking out the *pgi* gene in order to redirect carbon flux to the pentose phosphate pathway, enhance nucleotide synthesis and consequently increase pDNA production. GALG20 (MG1655*ΔendAΔrecAΔpgi*) produced 25-fold more pDNA (19.1 mg/g DCW) than its parental strain, MG1655*ΔendAΔrecA* (0.8 mg/g DCW), in glucose. For the first time *pgi* was identified as an important target for constructing a high-yielding pDNA production strain.

Introduction

Non-viral gene therapy is a promising approach for the treatment of genetic disorders and acquired diseases, and for the prevention of infectious diseases. The discovery that naked plasmid DNA (pDNA) could mediate gene transfer and expression *in vivo* (Wolff 1990) initiated the emergence of a new class of medicinal agents (Prazeres 2011). The efforts to develop such plasmid biopharmaceuticals have increased over the last several years, especially after the approval of the first veterinary DNA vaccines (Han 2009; Williams 2009). The relevance of non-viral gene therapy is underscored by the fact that plasmid DNA accounts for 25% of the vectors used in clinical trials (http://www.wiley.com//legacy/wileychi/genmed/clinical/).

One of the aims of research on plasmid biopharmaceuticals has been the development of *Escherichia coli (E. coli)* host strains capable of producing the large amounts of pDNA required for clinical trials and eventually commercialization (Bower and Prather 2009; Goncalves 2011; Lara and Ramirez 2012). Roughly speaking, volumetric pDNA titers lower than 250 mg/L are typically obtained in laboratory-scale fermentations (Prazeres 2011), whereas titers of the order of 1000-2100 mg/L have been reported in high-yield, preindustrial fermentations (Carnes 2006; Listner 2006; Phue 2008; Luke 2009; Williams 2009). On a dry cell weight (DCW) basis, the plasmid content of *E. coli* cells may be expected to vary anywhere between 0.5 and 5.0 % w/w, depending on the plasmid type, strain, growth conditions and growth phase (Prazeres 2011). *E. coli* host strains of the K-12 and B type with diverse mutagenized genetic backgrounds, such as DH5, DH5α, DH10B, JM108, JM101 and BL21, have all been used for pDNA production (Bower and Prather 2009; Carnes and Williams 2007; Cunningham 2009; Prather 2003; Williams 2009). However, most of these strains were originally developed to facilitate the cloning of heterologous genes and for the production of recombinant proteins, and thus may not be the most appropriate for pDNA

manufacturing. A more rational approach has been pursued recently, whereby genes are selected and mutated on the basis of *E. coli* central carbon metabolism and plasmid properties. This has led to the generation of a plethora of new pDNA production strains, such as DH5α*ΔfruR* (Ow 2007), BL21*ΔendAΔrecA* (Phue 2008), JM101*ΔpykFΔpykA* (Cunningham 2009), DH5α*zwf:rpiA*$^{+}$ (Williams 2009) and W3110*ΔPTSGalP*$^{+}$*ΔpykA*(Pablos 2011).

Reducing acetate and enhancing nucleotide production are some of the strategies that are used to increase pDNA yields. Knockouts of selected genes, such as *pykF, pykA, ackA-pta* and *poxB* have been explored to reduce acetic acid formation in pDNA production strains (Carnes 2011; Cunningham 2009; Pablos 2011). Genes in the pentose phosphate pathway such as *zwf* and *rpiA* have also been overexpressed in order to enhance nucleotide production for pDNA synthesis (Wang 2006; Williams 2009). However, most of these mutations were made in strains with highly mutagenized genetic backgrounds. Thus, it is unclear whether the specific strain background had an effect on the mutations introduced. In addition, these highly mutated strains exhibit a lower growth rate than parental (wild-type) strains, a characteristic that suggests a less healthy organism.

The trade-off between strain engineering and fermentation strategy is still a hurdle for the development of new pDNA production strains. Differences in fermentation strategies (batch or fed-batch) (O'Kennedy *et al.,* 2003; Ow *et al.,* 2007; Ow *et al.,* 2009), medium composition and carbon source (glucose or glycerol) (Oh and Liao 2000) were previously shown to affect *E. coli* metabolism and should also be taken into consideration when producing pDNA. It is well known that glycerol has the advantage of minimizing acetate formation in fermentation processes and it is becoming an inexpensive and attractive carbon source (Carnes 2006; Carvalho 2011). On the other hand, glucose has been used for several years in high-density cultures and for pDNA production and is both accessible and

inexpensive (Luli and Strohl 1990). High acetate formation has been observed with glucose; however, accumulation profiles depend on the strain (Phue 2005). *E. coli* strains have been engineered to optimize glucose uptake and reduce acetate formation (De Anda 2006). For example, the strain VH33 (PTS^-GalP^+), a derivative of W3110 with an inactive phosphotransferase system (PTS) and a strong promoter for *galP*, has shown improved production of pDNA (Pablos 2011; Soto 2011). Interestingly, high yield pDNA production was shown with strain BL21*ΔendAΔrecA* in both glucose and glycerol, while DH5α produced high pDNA yields only in glycerol (Phue 2008).

In this work, we set out to enhance pDNA production by rationally mutating key genes of the glycolytic pathway. We first chose genes which had already been shown to increase pDNA yields, *pykF* and *pykA* (Cunningham 2009; Pablos 2011), and deleted them in two different strains: MG1655*ΔendAΔrecA*, a nearly wild-type genetic background; and DH5α, a commonly used, but highly mutagenized strain. Deletions of *endA* and *recA* were made in MG1655 in order to minimize recombination and non-specific digestion of DNA (Phue 2008; Summers 1998). We also analyzed the influence of carbon source (glucose versus glycerol) among the different strains developed. Finally, we explored the effect of a previously untested mutation with the introduction of a new knockout, *pgi*, in MG1655*ΔendAΔrecA* to redirect carbon flow into the pentose phosphate pathway (PPP) and promote nucleotide synthesis.

Material and Methods

Strains and plasmids

The bacterial strains used in this study are indicated in Table 1. MG1655 was kindly donated by Professor Gregory Stephanopoulos of the Department of Chemical Engineering at the Massachusetts Institute of Technology (Cambridge, MA, USA). Gene deletions of *endA*,

recA, *pykF*, *pykA* and *pgi* in MG1655 and JM101were carried out with P1 transduction using the following strains from the Keio collection (Baba 2006) as donors: JW1666-1 [*ΔpykF751::kan*]; JW1843-1 [*ΔpykA779::kan*]; JW3985-1 [*Δpgi-721::kan*]; JW2912-1 [*ΔendA720::kan*] and JW2669-1 [*ΔrecA774::kan*]. The *recA* gene was always the last to be deleted, since P1 transduction requires an active RecA recombinase. The kanamycin cassette was removed using plasmid pCP20 as described by Datsenko and Wanner (2000), and successfully constructed mutant strains were verified by colony PCR using appropriate primers. The standard protocol for inactivation of chromosomal genes (Datsenko and Wanner 2000) was adapted to knock-out *pykF* and *pykA* in DH5α. Briefly, DH5α was transformed with a *recA* containing plasmid, pKD46*recA*+, and RecA recombinase expression was induced with arabinose. DH5α(pKD46*recA*+) was transformed with the specific PCR fragment generated using appropriate primers, and homologous recombinants were selected with kanamycin. The plasmid pKD46*recA*+ is temperature sensitive and was cured by raising the temperature. The remaining steps of the protocol were performed as described previously by Datsenko and Wanner (2000). All strains were transformed with plasmid pVAX1GFP (3697 bp), derived from Invitrogen's (Carlsbad, CA) pVAX1LacZ as described previously (Azzoni 2007).

Medium and growth conditions

Transformed strains were maintained on agar plates containing 30 µg/mL of kanamycin. A seed bank was prepared from single colonies picked from each plate and inoculated in Luria Bertani (LB) medium supplemented with 30 µg/mL of kanamycin. Cultures were grown to mid-exponential phase at 250 rpm, 37ºC and frozen at -80ºC in 15% (v/v) final concentration glycerol. The inoculum was prepared from frozen seed stocks in complex medium [Bacto peptone, 10 g/L; yeast extract, 10 g/L; $(NH_4)_2SO_4$, 3 g/L; K_2HPO_4, 3.5 g/L; KH_2PO_4, 3.5 g/L; thiamine, 199 mg/L; $MgSO_4$, 1.99 g/L; trace element solution, 1

mL/L (Listner 2006)] supplemented with 30 µg/mL of kanamycin, grown to mid-exponential phase and then used to inoculate batch cultures to an initial optical density at 600 nm (OD_{600}) of approximately 0.1. Cultures were grown at 37ºC for 24 hours in 250 mL shake flasks containing 50 mL of complex medium supplemented with 30 µg/mL of kanamycin, initial pH of 7.1 and aeration by shaking at 250 rpm. Glucose and glycerol were used as the primary carbon sources as indicated in the Results. Cells were sampled at 6, 12 and 24 hours to quantify glucose, glycerol and acetate, and at 12 and 24 hours to quantify pDNA.

Biomass quantification

Samples were taken every 3 hours to determine biomass concentration. OD_{600} was measured in a Beckman Coulter DU 800 spectrophotometer. Dry cell weight (DCW) was determined using a vacuum filtration system. 10 mL of each sample was filtered in previously weighed filter papers and then dried at 42ºC. A linear correlation was determined between cell concentration (g/L) and OD_{600nm}.

Plasmid DNA quantification

Plasmid DNA was quantified from crude lysates prepared from OD_{600nm} = 10 cell pellets using the method described by Listner (2006). The method was modified slightly: cell pellets were harvested by centrifugation at 5000 x *g* for 15 minutes, the 37°C incubation took place with 250 rpm shaking, and 5 µL of RNase A solution (10 mg/mL) was used per mL of lysate. The resulting lysates were analyzed using a Gen-Pak FAX anion-exchange column (Waters Corporation) on an Agilent 1100 Series HPLC system. The HPLC method was run at a constant flow rate of 0.75 mL/min and consisted of a 10-minute linear NaCl gradient from 300 mM to 660 mM, followed by 5.5 minutes at 1 M NaCl, after which the column was flushed with 0.04 M phosphoric acid for 4.5 minutes. Before the next sample injection, the column was equilibrated with 300 mM NaCl for 10 min. Plasmid DNA eluted at

approximately 610 mM NaCl and was detected at 260 nm with a diode array detector (DAD). A highly linear standard curve of pVAX1GFP was prepared using pDNA purified with the Hi-Speed QIAfilter Plasmid Maxi Kit (Qiagen) and quantified using absorbance at 260 nm on an Implen NanoPhotometer.

Metabolite quantification

To determine the concentration of glucose, glycerol and acetic acid, culture samples were centrifuged at 5000 x *g* for 15 minutes, and the aqueous supernatant was used for HPLC analysis on an Agilent 1100 Series HPLC system equipped with an Aminex HPX-87 H anion exchange column (Bio-Rad Laboratories) and refractive index detector (RID). The LC method was run at a constant flow rate of 0.6 mL/min with 5 mM H_2SO_4 as the mobile phase, at 50ºC for 25 min.

Statistical analyses

The effect of the gene knockouts was measured by pDNA yield quantification. T-tests for independent samples (SSPS Statistics) were done to identify significant differences in pDNA yields between parental and mutated strains. Differences were considered statistically significant when the *P* value was < 0.05. At least three independent duplicate experiments were conducted in order to confirm the results. Standard error of mean (SEM) was calculated for all measurements of pDNA yields, biomass, acetate and growth rate.

Results

Effect of carbon source on *pykF* and *pykA* knockout strains

Cuningham (2009) demonstrated the benefits of the double *pykF-pykA* mutations in a JM101 strain grown on glucose (Fig. 1). We chose to investigate these same mutations in the nearly wild-type MG1655*ΔendAΔrecA* and in the commonly used DH5α strain, using either

glycerol or glucose as the primary carbon source. The first experiments were conducted with an initial concentration of glycerol and glucose of 20 g/L and with MG1655*ΔendAΔrecA,* GALG1011 (MG1655*ΔendAΔrecAΔpykFΔpykA*), DH5α and DH5α*ΔpykFΔpykA,* all carrying pVAX1GFP. MG1655*ΔendAΔrecA* and DH5α grew faster than their respective *pykF* and *pykA* double knockout mutant strains in both glucose and glycerol. However, higher biomass content was obtained in glycerol. GALG1011 produced less acetate than MG1655*ΔendAΔrecA* in both carbon sources. In contrast, DH5α and DH5α*ΔpykFΔpykA* produced similar amounts of acetate. In general, less acetate was obtained in glycerol for all strains (Table 2).

The double knockout of *pykF* and *pykA* had a negative impact on pDNA production in both strains when using glycerol as carbon source (Table 3). On the other hand, the double knockout increased pDNA yield 2-fold in GALG1011 *vs.* MG1655*ΔendAΔrecA* when glucose was the main carbon source. In glucose, the phosphotransferase system (PTS) is able to couple pyruvate formation with carbohydrate transport, partially mitigating the effect of *pyk* deletions. However, the PTS system is not utilized for glycerol uptake and hence this additional source of pyruvate is not available (Oh and Liao 2000). Nevertheless, pDNA yields were significantly lower in glucose than in glycerol for all Pyk mutant strains when the initial concentration of both carbon sources was 20 g/L (Table 3). It has been reported that 10 g/L of glucose can inhibit pDNA synthesis in *E. coli* B (Zhi-nan 2005) and that *E. coli* K-12 grows slower and accumulates more acetate than B strains at high glucose concentrations (Phue 2005). Therefore, inhibition of pDNA synthesis could also be expected in K-12 strains with high glucose. Of the strains and conditions examined, MG1655*ΔendAΔrecA* was the best producer of pDNA in glycerol, although it produced the highest quantity of acetate (Tables 2 and 3). It is known that acetate secretion can shuttle carbon away from nucleotide

synthesis which could be disadvantageous for pDNA formation, but the exact effect of acetate on pDNA production is not yet elucidated (Carnes 2011; Wang 2006).

Glucose inhibition in plasmid DNA production

To determine the effect of glucose concentration on pDNA production, MG1655*ΔendAΔrecA* and GALG1011 bearing pVAX1GFP were grown in three different conditions: 5 g/L of glucose initially plus 10 g/L of glucose after 12 hours (5+10 g/L), 10 g/L of glucose initially plus 10 g/L of glucose after 12 hours (10+10 g/L), and 20 g/L with no extra addition of glucose. MG1655*ΔendAΔrecA* and GALG1011 produced 5-fold and 4-fold more pDNA, respectively, in (5+10 g/L) than 20 g/L of glucose (Table 3). However, when the initial glucose concentration was 5 g/L, the difference in pDNA specific yield (mg/g DCW) between these strains was less than 2-fold (Table 3). In (5+10 g/L) glucose, GALG1011 still produced less acetate and grew slower than MG1655*ΔendAΔrecA* (Table 2). Similar biomass was formed for both strains, and the final biomass achieved in (5+10 g/L) glucose was comparable to that obtained in glycerol, though the pDNA yield was still higher in glycerol. Experiments were also performed with glucose addition after 9 hours, but no difference was observed in pDNA yields (data not shown).

After determining the best glucose concentration, (5+10 g/L), for pDNA production in MG1655*ΔendAΔrecA* and GALG1011, we tested DH5α and DH5α*ΔpykFΔpykA,* in order to determine whether the negative performance of the double mutant with 20 g/L glucose (Table 3) was due to inhibition or to strain genetic background. pDNA production increased 2-fold in DH5α when moving from 20 g/L glucose to (5+10 g/L) glucose. However, the negative effect of *pykF* and *pykA* knockouts was maintained. Therefore, it appears that the genetic background of DH5α is suppressing the positive effects of *pykF* and *pykA* gene knockouts observed in GALG1011.

Effect of strain background on *pykF* and *pykA* double knockouts

As stated previously, the beneficial effects of the *pykF-pykA* double knockout for pDNA production were demonstrated in JM101 grown in glucose (Cunningham 2009). There are no reports about the behavior of JM101*ΔpykFΔpykA* bearing plasmid in glycerol. To further examine the effect of strain genetic background, we decided to construct JM101*ΔpykFΔpykA* and to test it in the same rich medium and conditions as GALG1011 and DH5α*ΔpykFΔpykA*. The loss of Pyk is expected to reduce acetate accumulation, and glycerol is frequently used for the same purpose. Thus, the double mutations are not expected to favor pDNA production in glycerol over the unmutated strain; however, because this carbon source was not previously tested, it is unclear if the effect of the mutations would be negative or neutral. Consistent with the results presented by Cunningham (2009), JM101*ΔpykFΔpykA* produced 2-fold more pVAX1GFP (5.3 or 2.5 mg/g DCW) than JM101 (2.6 or 1.3 mg/g DCW) when grown in 5 or 20 g/L glucose, respectively. However, as with the other engineered strains, JM101*ΔpykFΔpykA* (2.2 mg/g DCW) produced less pDNA than JM101 in glycerol-rich medium (8.4 mg/g DCW; Table 3). In general, the double knockout of *pykF* and *pykA* was effective in MG1655*ΔendAΔrecA* and JM101 in glucose, but it was not beneficial in DH5α. The highly mutagenized genetic background of DH5α appears to affect the *pykF* and *pykA* mutation.

Effect of single knockouts of *pykF* or *pykA* gene

The previous results showed that the *pykF* and *pykA* double knockout can be beneficial in glucose and that the mutation had a positive effect in MG1655*ΔendAΔrecA* and JM101 (Table 3). Nevertheless, the *pykF* and *pykA* mutant strain GALG1011 still produced much less plasmid in glucose (6.6 mg/g DCW) than the nearly wild-type strain, MG1655*ΔendAΔrecA,* in glycerol (11.2 mg/g DCW), (Table 3).

In an effort to increase pDNA yields, we decided to investigate *pykF* or *pykA* single knockouts in MG1655*ΔendAΔrecA*. A single knockout could be more effective than the double knockout for pDNA production in glucose because more pyruvate would be formed and higher ATP yields would be obtained through glycolysis. At the same time, carbon flux would be boosted to the pentose phosphate pathway, increasing nucleotide synthesis (Siddiquee 2004). For example, Pablos (2011) showed that a single *pykA* knockout increased pDNA production 3-fold in W3110*ΔPTSGalP*$^{+}$ in glucose.

To determine the effect of a *pykF* or *pykA* single knockout in MG1655*ΔendAΔrecA,* we generated strains GALG10 (MG1655*ΔendAΔrecAΔpykF*) and GALG11 (MG1655*ΔendAΔrecAΔpykA*) and tested them in glucose and glycerol under the same conditions as described previously (Table 3). In both carbon sources, one single mutation was found to be more effective for pDNA production than the double mutation. In glycerol, MG1655*ΔendAΔrecA,* GALG10 and GALG11 produced similar amounts of pDNA (11.2, 8.6 and 10.3 mg/g DCW). In contrast, GALG10 (10.5 mg/g DCW) and GALG11 (13.1 mg/g DCW) produced more pDNA than MG1655*ΔendAΔrecA* (3.6 mg/g DCW) or GALG1011 (6.6 mg/g DCW) in glucose. GALG11 achieved high pDNA yields in glucose (13.1 mg/g DCW), even in comparison with the yields obtained with MG1655*ΔendAΔrecA* in glycerol (11.2 mg/g DCW), (Table3). Nevertheless, both GALG10 and GALG11 produced little pDNA when the initial concentration of glucose was 20 g/L (≤1 mg/g DCW).

Single mutations in *pykF* or *pykA* genes were also constructed in DH5α, generating the strains DH5α*ΔpykF* and DH5α*ΔpykA.* Surprisingly, DH5α*ΔpykF* (5.9 mg/g DCW) produced more pDNA than DH5α (1.8 mg/g DCW) in glucose. On the contrary, DH5α*ΔpykA* (0.9 mg/g DCW) displayed a similar behaviour to DH5α*ΔpykFΔpykA* (0.3 mg/g DCW) and produced much less pDNA. Although the *pykF* single knockout had positive effects in DH5α,

the final pDNA yields obtained with DH5α and DH5α*ΔpykF* bearing pVAX1GFP were lower than the ones obtained with GALG10 and GALG11 (Table 3).

Effect of *pgi* knockout on plasmid DNA production

The main advantages of removing pyruvate kinase (Pyk) in a pDNA production strain through the deletion of both *pykF* and *pykA* genes would be the reduction of acetate and increase of carbon flux into the pentose phosphate pathway (PPP). On the other hand, the total lack of Pyk could reduce energy available for the cells by reducing the ATP content. In this work, we have shown that deletion of both pyruvate kinase isozymes increased pDNA production in the parental strain MG1655*ΔendAΔrecA* in glucose. However, higher amounts of pDNA were obtained when only pyruvate kinase I or II were absent in the parental strain in glucose, a result that suggests the importance of generating some pyruvate and completing glycolysis.

To increase the pentose phosphate pathway flux without compromising the energy obtained from the generation of pyruvate, we tried a different strategy in order to enhance nucleotide production and reduce glycolytic flux at the same time (Fig. 1). It has been reported that strains carrying high copy pDNA require extra synthesis of nucleotides and that the carbon flux into the PPP may not be sufficient to meet cellular needs (Flores 2004). The elimination of phosphoglucose isomerase (*pgi*) aims to redirect the carbon flux into the PPP, enhance synthesis of nucleotides, and also provide high amounts of reducing cofactors (i.e., NADPH). Pgi mutant strains were recently reported to improve the production of xylitol (Chin and Cirino 2011; Chin 2009), chiral compounds for the pharmaceutical market (Siedler 2011), and second generation biofuels (Yao 2011).

GALG20 (MG1655*ΔendAΔrecAΔpgi*) produced 3-fold more pVAXGFP pDNA (11.6 mg/g DCW) than MG1655*ΔendAΔrecA* in (5+10 g/L) glucose (3.6 mg/g DCW). In glycerol,

GALG20 produced similar amounts of pDNA as MG1655*ΔendAΔrecA* (Fig. 2). Surprisingly, this strain had the best performance in 20 g/L of glucose, producing 25-fold more pDNA (19.1 mg/g DCW) than the parental strain (0.8 mg/g DCW), and 46% or 65 % more, respectively, than the previously best identified performers, GALG11 in 5+10 g/L glucose (13.1 mg/g DCW) and MG1655*ΔendAΔrecA* in 20 g/L glycerol (11.5 mg/g DCW).

Discussion

The *de novo* design of *E. coli* strains specifically for pDNA amplification is expected to enhance production yields when compared to strains with highly mutagenized genetic backgrounds. In this study, we performed a series of mutations in the wild type MG1655 strain and in the laboratory strain DH5α in order to improve pDNA yields. The genes coding for pyruvate kinase (*pykA*, *pykF*) and phosphoglucose isomerase (*pgi*) were selected as targets due to their role in the PPP (Fig. 1).

In order to reduce acetate and increase carbon flux into the PPP, a double knockout of *pykF* and *pykA* was first created. Our results show that the strain genetic background can indeed interfere in the outcome of mutations which are otherwise rationally designed. Of the Pyk mutant strains examined, the strains derived from the nearly wild type MG1655*ΔendAΔrecA* (GALG10 and GALG11) were the best producers of pDNA, whereas DH5α*ΔpykFΔpykA* and DH5α*ΔpykA* were the worst producers of pDNA in glucose (Table 3). The negative effect of the *pykF* and *pykA* double knockout on the production of pDNA in DH5α could be ascribed to the interaction between the strain genotype and the new mutations added to the strain, though it is not clear which specific mutations result in the negative phenotype. In contrast, DH5α*ΔpykF* produced more plasmid than DH5α in glucose, a result that points to an essential role of the *pykA* gene in DH5α. Although, DH5α*ΔpykF* had

increased pDNA yields when compared to DH5α, the obtained yields were lower than those obtained with GALG10 and GALG11. Other advantages associated with the use of the nearly wild-type strain MG1655*ΔendAΔrecA* as a starting point for strain engineering instead of DH5α include a more robust growth and a lack of auxotrophies that can benefit pDNA production.

Strains with single mutations of either *pykF* or *pykA* were found to be more efficient in producing pDNA than strains containing the double mutation. The synthesis of pDNA was likely more advantageous when the pyruvate kinase activity was reduced as opposed to abolished because glycolytic flux was down-regulated while still enabling additional ATP production and up-regulation of glucose flux through the PPP. For example, Siddiquee (2004) demonstrated that a *pykF* single knockout in *E. coli* increased glucose flux through the PPP. Moreover, linear programming has predicted different carbon fluxes for *E. coli* lacking pyruvate kinase, resulting in different ATP yields (Phalakornkule 2001). Since cells, as living organisms, are difficult to phenotypically predict, different strains (MG1655 and DH5α) with the same gene knockout (*pykA* or *pykF*) can have diverse carbon fluxes and result in different ATP yields, favoring pDNA synthesis or not.

The abolishment of the *pgi* gene should redirect glucose-6-phosphate preferentially to the PPP, but glycolysis would continue due to the generation of fructose-6-phosphate and glyceraldeyde-3-phosphate (Fig. 1). In general, high amounts of NADPH, nucleotides and ATP would be obtained. Indeed, the inactivation of the *pgi* gene in *E. coli* previously resulted in the redirection of glucose predominantly via the PPP, but a minor fraction of glucose was still catabolized in the Entner-Doudoroff Pathway (EDP) (Canonaco 2001). A large excess of NADPH was detected in Pgi mutant strains (Canonaco 2001; Chin and Cirino 2011; Siedler 2011), which was shown to improve biotransformations in various processes. However, an excess of NADPH can cause a redox imbalance in the cell, imposing stress that may appear

as a large reduction in growth rate (Charusanti 2010). In the specific case of pDNA synthesis, a mathematical model has demonstrated that high generation of NADPH would be effective for increasing yields (Cunningham 2009). Nevertheless, no Pgi mutant strain has been previously reported for the purpose of producing pDNA.

Together the benefits of increasing glucose flux into the PPP, such as enhancement of nucleotides and of NADPH generation, turn a Pgi mutant strain into a high potential pDNA production strain. In fact, GALG20 produced 25-fold more pDNA than its parental strain in 20 g/L of glucose. A previous study has shown that catabolite repression can be relaxed by *pgi* mutants (Yao 2011). Indeed, large amounts of biomass (8.8 g/L) and pDNA (19.1 mg/g DCW) and low amounts of acetic acid (0.02 g/L) were formed by GALG20 in high concentrations of glucose. Another advantage of GALG20 for pDNA production was that the growth rate was similar to the parental strain in glucose (Table 2), whereas a previous study demonstrated a significant reduction of growth rate in a *pgi* mutant strain grown in the same carbon source (Ahn 2011). The differences in the behavior of these two *pgi* mutants in glucose could be associated with the strain genetic background between the two studies, *E. coli* MG1655*ΔendAΔrecA* and KPM SA1 (*ΔaraLΔaraK*), respectively. In the current work, we have demonstrated that strain background can substantially influence the effect of gene knockouts, such as the differences between the *pykF* and *pykA* mutations in MG1655*ΔendAΔrecA* versus DH5α.

In the *pgi* mutant strain, the level of glucose-6-phosphate is significantly higher, which can destabilize the PTS system and in turn, alter the level of cyclic AMP (cAMP) (Morita 2003). On the other hand, *pgi* mutant cells up-regulate the catabolite repressor activator (Cra), due to the decrease in fructose 6-phosphate (Yao 2011). Nevertheless, it is not clear yet how cAMP or Cra mechanisms of gene regulation would correlate with pDNA synthesis. Previous studies suggested that increasing cAMP levels could be associated with

enhancement of pDNA yields (Cunningham 2009), and the elimination of Cra was shown to increase pDNA (Ow 2009). Further work would be necessary to elucidate the role played by cAMP and Cra in plasmid DNA synthesis, and *pgi* mutant cells could be a useful target to study metabolic network behavior associated with pDNA production. The regulation of glucose-6-phosphate-1-dehydrogenase (Zwf) or other enzymes in the PPP could limit pDNA synthesis. Based on previous evidence (Wang 2006), ribose phosphate isomerase (Rpi) could also limit nucleotide production because the relative amount of ribose 5-phosphate (R5P) and xylose 5-phosphate (Xu5P) depends on cellular needs. Overexpression of Zwf and Rpi would be potential alternatives to increase pDNA yields in *pgi* mutant cells.

Among the strains generated and analyzed we selected the top three pDNA producer strains and compared their performance with the commonly used lab strain, DH5α (Table 4). GALG20 and GALG11 performed better in glucose while MG1655*ΔendAΔrecA* and DH5α performed better in glycerol. The *pgi* mutation in the MG1655*ΔendAΔrecA* wild-type strain resulted in the best volumetric (140.8 mg/L) and specific (19.1 mg/g DCW) pDNA yields. To our knowledge, this is the highest reported value for specific plasmid yield for a constant temperature batch process. This figure is only comparable to the 19.2 mg/g DCW obtained by Ow (2009) in a fed-batch system with a DH5αΔFruR strain at 37ºC. Further experiments will be carried out and different fermentation strategies will be explored in order to investigate the true potential of these high pDNA producer strains.

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Table 1 Bacterial strains used in this study.

Strain	Genotype	Reference
MG1655*ΔendAΔrecA*	*F⁻ λ ⁻ilvG rfb-50 rph1ΔendAΔrecA*	Prather Lab
GALG10	MG1655*ΔendAΔrecAΔpykF*	This study
GALG11	MG1655*ΔendAΔrecAΔpykA*	This study
GALG1011	MG1655*ΔendAΔrecAΔpykF ΔpykA*	This study
DH5α	F- ö80*lac*ZÄM15 Ä(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 ë⁻	Invitrogen
DH5α*ΔpykA*	DH5α *ΔpykA*	This study
DH5α*ΔpykF*	DH5α *ΔpykF*	This study
DH5α*ΔpykFΔpykA*	DH5α *ΔpykFΔpykA*	This study
JM101	F´ *traD36 proA⁺B⁺ lacI^q* Δ*(lacZ)M15/* Δ*(lac-proAB) glnV thi*	ATCC33876
JM101*ΔpykFΔpykA*	JM101*ΔpykFΔpykA*	This study
GALG20	MG1655*ΔendAΔrecAΔpgi*	This study

Table 2 Biomass, acetate and growth rate in glucose versus glycerol for parental strains versus Pyk and Pgi mutant strains.

Strain	**Carbon Source**	**Biomass** (g/L)	**Acetate** (g/L)	**Growth rate** (h^{-1})
MG1655*ΔendAΔrecA*	Glycerol 20 g/L	7.7 ± 0.8	3.86 ± 0.01	0.60 ± 0.01
GALG1011		8.6 ± 0.9	0.08 ± 0.01	0.49 ± 0.01
GALG20		8.5 ± 0.5	0.61 ± 0.04*	0.60 ± 0.05
DH5α		11.3 ± 0.9	0.05 ± 0.01	0.55 ± 0.01
DH5α*ΔpykFΔpykA*		9.0 ± 0.6	0.04 ± 0.01	0.31 ± 0.01
MG1655*ΔendAΔrecA*	Glucose 20 g/L	3.5 ± 0.3	5.74 ± 0.04	0.65 ± 0.01
GALG1011		4.3 ± 0.2	4.81 ± 0.03	0.53 ± 0.01
GALG20		10.9 ± 0.2	1.55 ± 0.02*	0.77 ±0.05
DH5α		3.8 ± 0.2	4.72 ± 0.04	0.49 ± 0.08
DH5α*ΔpykFΔpykA*		3.0 ± 0.4	4.21 ± 0.02	0.28 ±0.01
MG1655*ΔendAΔrecA*	Glucose 5+10 g/L	11.8 ± 0.3	1.98 ± 0.05*	0.55 ± 0.08
GALG1011		12.2 ± 0.2	0.64 ± 0.05*	0.40 ± 0.10
GALG10		9.8 ± 0.4	0.86 ± 0.05*	0.30 ± 0.1
GALG11		12.0 ± 0.4	1.23 ± 0.05*	0.48 ± 0.05
GALG20		9.6 ±0.2	0.68 ± 0.05*	0.67 ± 0.05

Strains were grown in shake flasks at 37ºC. Average value ± standard error of mean (SEM) is shown.

*Values corresponding to hour 8, since at hour 24 no acetate was detectable.

Table 3 Effect of glucose and glycerol in different concentrations on plasmid DNA production for different strains.

Strain	Carbon Source (g/L)		pDNA yield (mg/gDCW)	pDNA yield (mg/L)
MG1655*ΔendAΔrecA*	Glucose	20	0.8 ± 0.1	1.5 ± 0.1
		10+10	0.8 ± 0.1	1.9 ± 1.2
		5+10	3.6 ± 0.7	27.4 ± 1.9
	Glycerol	20	11.5 ± 0.8	79.3 ± 1.4
		5+10	11.2 ± 0.5	75.1 ± 3.9
GALG1011	Glucose	20	1.6 ± 0.3	3.2 ± 0.4
		10+10	1.2 ± 0.5	4.3 ± 1.7
		5+10	6.6 ± 0.4	42.1 ± 3.5
	Glycerol	20	7.6 ± 1.1	50.5 ± 1.3
		5+10	2.7 ± 0.2	20.9 ± 0.2
GALG10	Glucose	20	0.99 ± 0.1	3.45 ±0.3
		5+10	10.5 ± 1.3	81.5 ± 5.7
	Glycerol	5+10	8.6 ± 0.6	69.2 ± 3.9
GALG11	Glucose	20	0.65 ± 0.1	2.4 ± 0.3
		5+10	13.1 ± 0.2	94.1 ± 2.7
	Glycerol	5+10	10.3 ± 0.6	79.4 ± 7.0
GALG20	Glucose	20	19.1 ± 1.5	140.8 ± 0.8
		5+10	11.6 ± 1.1	88.9 ± 0.7
	Glycerol	5+10	10.1 ± 0.1	65.5 ± 1.4
DH5α	Glucose	20	0.8 ± 0.1	1.3 ± 0.1
		5+10	1.8 ± 0.7	9.6 ± 0.5
	Glycerol	20	4.4 ± 0.3	34.7 ± 0.6
DH5α*ΔpykFΔpykA*	Glucose	20	0.4 ± 0.1	2.6 ± 0.2
		5+10	0.3 ± 0.3	0.9 ± 0.2
	Glycerol	20	1.5 ± 0.3	5.9 ± 1.1
DH5α*ΔpykF*	Glucose	5+10	5.9 ± 0.1	36.9 ± 0.4
DH5α*ΔpykA*	Glucose	5+10	0.9 ± 0.1	1.5 ± 0.1
JM101	Glucose	20	1.3 ± 0.2	4.5 ± 0.8
		5	2.5 ± 0.4	35.5 ± 6.8
	Glycerol	20	8.4 ± 1.4	54.3 ± 2.8
JM101 *ΔpykFΔpykA*	Glucose	20	2.6 ± 0.3	12.5 ± 2.8
		5	5.3 ± 1.7	28.5 ± 4.3
	Glycerol	20	2.2 ± 1.3	12.5 ± 0.5

Strains were grown in shake flasks at 37ºC. Average value ± standard error of mean (SEM) is shown.

Table 4 Top three high-yield pDNA production strains identified in this study versus a common strain for pDNA production, DH5α.

Strain	Carbon source	pDNA yield	
		volumetric (mg/L)	Specific (mg/g DCW)
GALG20	Glucose	140.8 ± 0.8	19.1 ± 1.5
GALG11	Glucose	94.1 ± 2.7	13.1 ± 0.2
MG1655*ΔendAΔrecA*	Glycerol	79.3 ± 1.4	11.5 ± 0.8
DH5α	Glycerol	34.7 ± 0.6	4.4 ± 0.3

Strains were grown in shake flasks at 37ºC. Average value ± standard error of mean (SEM) is shown.

Figure legends

Fig. 1 Gene knockout strategies to improve plasmid DNA production in *E. coli.* The knockout of the phosphoglucose isomerase (*pgi*) gene redirects glycolytic flux, increasing fluxes in the pentose phosphate pathway and enhancing nucleotide synthesis and NADPH production. Glycolysis is down-regulated, but proceeds via the formation of fructose 6-phosphate and glyceraldehyde 3-phosphate. The knockouts of pyruvate kinase genes *pykF* and *pykA* reduce acetate formation and increase fluxes in the pentose phosphate pathway.This figure represents a simplified version of *E. coli* central metabolism. Dark arrows represent high carbon flow in the pentose phosphate pathway, light arrows represent downregulation of glycolysis, and blank arrow represent null carbon flow. Abbreviations: G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; FDP, fructose 1,6-diphosphate; G3P, glyceraldehyde 3-phosphate; PEP, phosphoenolpyruvate; 6GPC, 6-phosphogluconate; RU5P, ribulose 5-phosphate; XU5P, xylulose 5-phosphate; R5P, ribose 5-phosphate; S7P, sedoheptulose 7-phosphate; E4P, erythrose 4-phosphate; PTS, phosphotransferase system.

Fig. 2 Effect of *pgi* knockout on pDNA specific yield (mg/g DCW) using MG1655*ΔendAΔrecA* grown in glycerol and glucose. Strains were grown for 24 h in shake flasks (37ºC, 250 rpm) with rich medium supplemented with 5+10 g/L of glucose (white bars), 5+10 g/L glycerol (gray bars) and 20 g/L of glucose (solid bars). Differences between MG1655*ΔendAΔrecA* and GALG20 were statistically significant ($p<0.05$) in different concentrations of glucose, but they were not statistically significant ($p>0.05$) in glycerol. The standard error of the mean (SEM) was calculated is represented with error bars.

Figure 1
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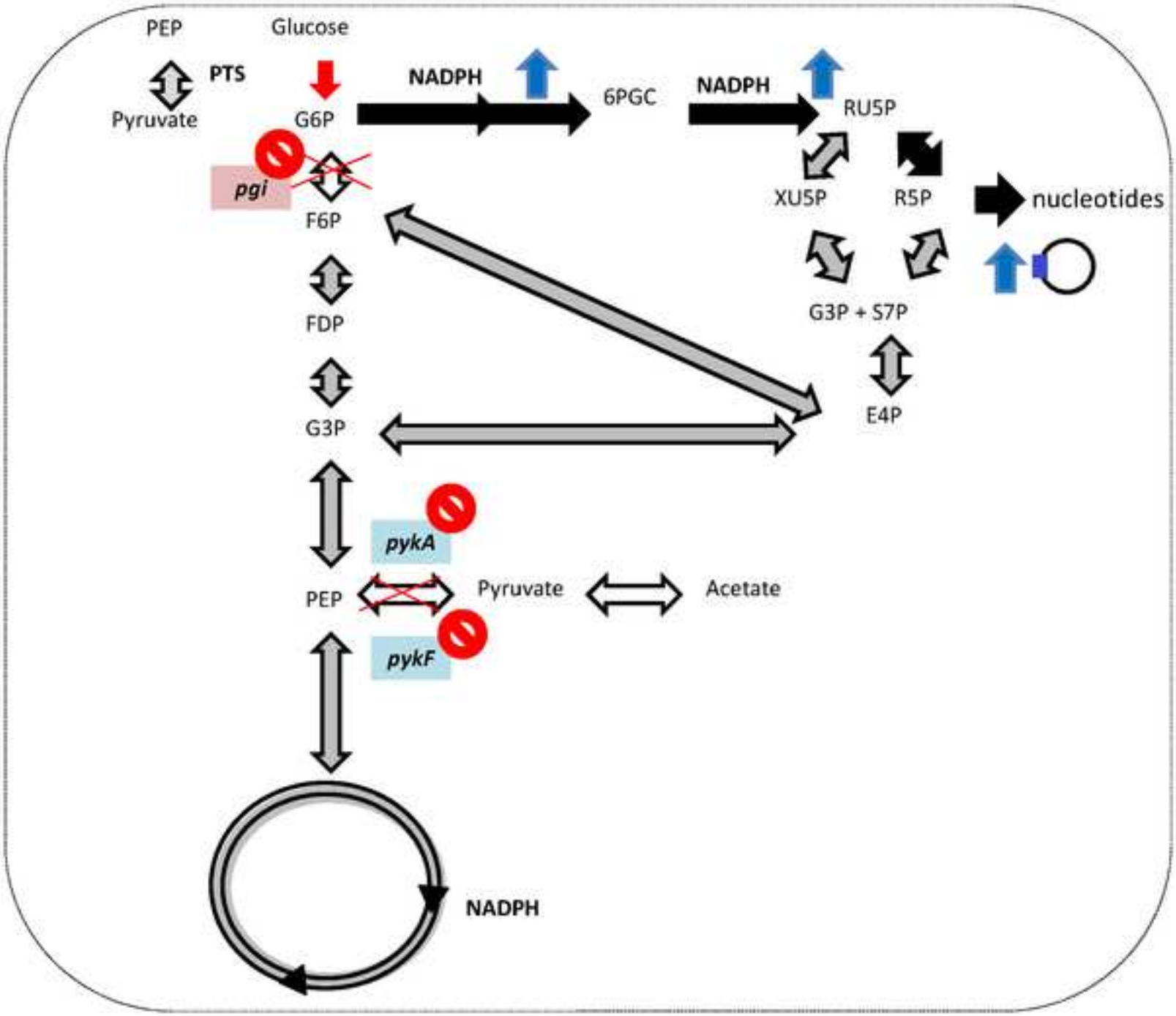

Figure 2
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