Biocatalysis and Agricultural Biotechnology 8 (2016) 45–49

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

# Genetic construction of recombinant *Pseudomonas chlororaphis* for improved glycerol utilization ☆

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ARTICLE INFO

*Article history:*
Received 7 March 2016
Received in revised form
6 June 2016
Accepted 1 August 2016
Available online 8 August 2016

*Keywords:*
Glycerol metabolism
Biodegradable polymer
Biosurfactant

ABSTRACT

This study is to use genetic engineering to improve the glycerol metabolic capability of *Pseudomonas chlororaphis* which is capable of producing commercially valuable biodegradable poly(hydroxyalkanoate) (PHA) biopolymers and biosurfactant rhamnolipids (RLs). In the study, the glycerol uptake facilitator or aquaglyceroporin gene (*glpF*) and the glycerol kinase (*glpK*) gene were PCR-cloned from *E. coli*, inserted into a shuttle vector pBS29P2-*gfp*, and expressed in *P. chlororaphis* by a *Pseudomonas* promoter P2. The *P. chlororaphis* recombinants were then tested for cell growth and glycerol metabolism in chemically defined medium containing 0.5% and 1.0% (v/v) glycerol. The simultaneous expression of *glpF* and *glpK* resulted in a shorter lag time for cell growth and a more immediate glycerol consumption by *P. chlororaphis*. In conclusion, the recombinant *P. chlororaphis* that grows more efficiently in glycerol is expected to improve the technoeconomics of PHA and RL production using the surplus bioglycerol byproduct stream from biodiesel production.



## 1. Introduction

*Pseudomonas chlororaphis* is a valuable microorganism that is capable of producing many industrially useful products. The organism is most valued as a nonpathogenic organism capable of producing the biologically active rhamnolipid biosurfactant (Solaiman et al., 2015). It can also produce the biodegradable medium-chain-length poly(hydroxyalkanoate) biopolymers (Solaiman et al., 2014). Furthermore, *P. chlororaphis* is a producer of several potent antifungal compounds against soil-borne plant-pathogenic fungi (Calderón et al., 2015). Commercial feasibility of *P. chlororaphis* to produce these microbial bioproducts, however, hinges on favorable techno-economic bioprocesses. Since the cost of fermentation feedstocks is a major contributor to the total bioproduction cost (Henkel et al., 2012; Ashby et al., 2013), many low-cost byproducts (*i.e.*, glycerol from biodiesel production) have been studied as fermentation substrates in order to help contain production costs of microbial bioproducts (Solaiman et al., 2006).

Genetic engineering is widely practiced to improve the efficiency of many bioprocesses that utilize various waste-streams as fermentation feedstocks. In the case of glycerol utilization in *E. coli*, recent advances have been documented in Mattam et al. (2013). Similar research in non-*E. coli* organisms, however, is much less extensive. In the *Pseudomonads*, for example, only the genetic system and biochemical pathway for glycerol metabolism in *Pseudomonas aeruginosa* was extensively studied (Schweizer et al., 1997). Even in this case, genetic engineering has not been reported to further enhance its glycerol utilization capability. In view of the potential industrial importance of *P. chlororaphis*, in this paper we report our study to genetically engineer a *Pseudomonas* species to better utilize glycerol as a sole carbon source for fermentation growth.

## 2. Material and methods

### 2.1. Microorganisms, culture media, and plasmids

*E. coli* strains were variously purchased from New England Biolabs (Ipswich, MA), Invitrogen (Carlsbad, CA), and Clonetech Laboratories (Mountain View, CA). *P. chlororaphis* NRRL B-30761 (Gunther et al., 2007) was from the ARS Culture Collection (Peoria, IL). Bacteria were routinely grown in LB medium at 30 °C (for *P. chlororaphis*) or 37 °C (for *E. coli*) with shaking (200–250 rpm). Cell growth and glycerol consumption by *P. chlororaphis* recombinant strains were characterized in a Mineral Salts Medium (MSM) supplemented with different amounts of glycerol (MSM+glycerol). (See Solaiman et al. (2015) for the composition of MSM.) The expression vector, pBS29P2-*gfp*, was previously described

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http://dx.doi.org/10.1016/j.bcab.2016.08.001
1878-8181/

**Table 1**
PCR amplicons and primers in this study.

| Amplicon (length) | Primer | |
|---|---|---|
| | Name | Sequence (5′-to-3′) |
| *glpF* (0.9 kb) | CL16–198-glpF-FW | CCCAAGAGCCGTAATATGAGTCAAACATCAAC |
| | CL16–198-glpF-RV | CAATCAGGATCCAATTTACAGCGAAGCTTTTTGTTC |
| *glpK* (1.5 kb) | CL16–198-glpK-FW | CCCAAGAGCCGTAATATGACTGAAAAAAAATATATC |
| | CL16–198-glpK-RV | CAATCAGGATCCAATTTATTCGTCGTGTTCTTCCCAC |
| *glpD* (1.5 kb) | CL16–198-glpD-FW | CCCAAGAGCCGTAATATGGAAACCAAAGATCTG |
| | CL16–198-glpD-RV | CAATCAGGATCCAATTTACGACGCCAGCGATAA |
| *glpFK* (2.4 kb) | CL16–198-glpF-FW | (see above) |
| | CL16–198-glpK-RV | (see above) |

(Solaiman and Swingle, 2010). Culture media were supplemented with tetracycline (at 12 μg ml$^{-1}$; $Tc_{12}$) when growing recombinant bacteria harboring pBS29P2-*gfp* plasmid or its derivatives.

### 2.2. Molecular cloning procedures

All routine molecular biology procedures were performed based on protocols described in Ausubel et al. (1987). Plasmid and genomic DNAs were isolated using the GenElute Plasmid Prep Kit and the GenElute Bacterial Genomic DNA Kit (both from Sigma-Aldrich), respectively. Genes of interest were PCR-amplified from genomic DNA and spliced into an expression vector using an In-Fusion HD Cloning Plus Kit (Clontech Laboratories). Sequences of PCR primers were based on those described by Rittmann et al. (2008). Recombinant plasmids were constructed in *E. coli*. These were then used to transform *P. chlororaphis* by electroporation and screened by GFP-based fluorescence measurement on a 96-well plate (Solaiman and Swingle, 2010). Nucleic acid sequence confirmation was carried out using an Applied Biosystems 3730 DNA Analyzer (Life Technologies Corp, Carlsbad, CA).

### 2.3. Characterization of P. chlororaphis recombinants

The seed cultures were grown in 3–5 ml of LB broth containing Tc (*i.e.*, LB/$Tc_{12}$) in 15-ml Falcon tubes at 30 °C and 200 rpm shaking for 3 days. These were used to inoculate 100–150 ml of LB/$Tc_{12}$ in 500-ml Erlenmeyer flasks. These cultures were grown for 16–18 h at 30 °C and 200 rpm, and the cells were then harvested by centrifugation (4 °C, 4000–4500 g, 10 min), washed with MSM + glycerol, and resuspended in 10–15 ml of the same medium. The cell suspensions (*ca.* 5 ml) were used to inoculate sets of triplicate 200 ml of MSM + glycerol (in 500-ml or 2-l capacity Erlenmeyer flasks as specified). The cultures were grown at 30 °C with 200 rpm rotary shaking for 2–3 days. Culture samples were periodically taken to monitor cell growth and glycerol consumption. Cell growth was monitored by measuring the absorbance of 1/10-diluted culture samples at 600 nm ($A_{600\ nm}$).

Glycerol concentration in the culture samples (stored at −20 °C until use) was assayed using a Free Glycerol Determination Kit (Sigma-Aldrich). Thawed culture samples were centrifuged (13,500 g, 3 min, ambient room temperature or R.T.), and the supernatant was diluted (at 1/10- to 1/20-dilution) before being added to the Free Glycerol Reagent (Sigma-Aldrich) for assay reaction. The Free Glycerol Reagent (200 μl) was introduced first to each well of a clear-bottom microtitre plate, and 1–2 μl of the diluted cell-free culture sample was then added to the reagent to initiate the reaction. Glycerol Standard Solution (Sigma-Aldrich; 0.26 mg glycerol ml$^{-1}$) was used (at 1–5 μl) to construct a calibration curve. After 15 min at R.T., absorbance at 540 nm ($A_{540\ nm}$) was recorded on a Thermo Labsystem Multiskan MCC/340 plate reader (Fisher Scientific).

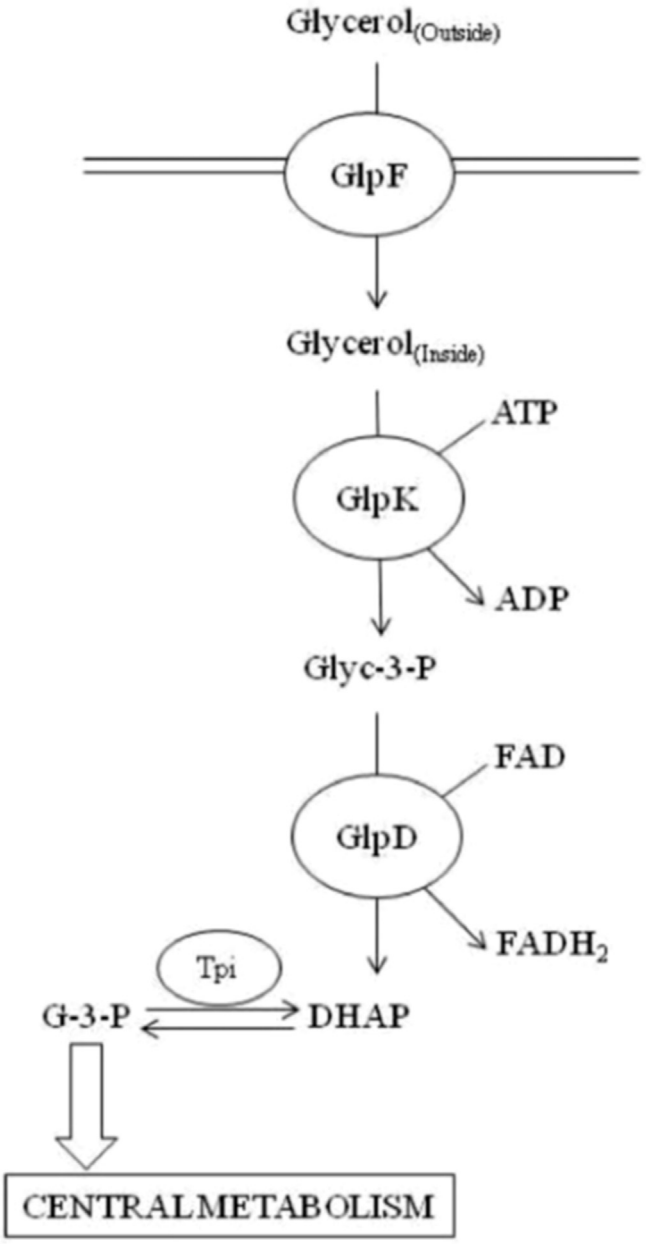


**Fig. 1.** Schematic of aerobic glycerol assimilation pathway. Abbrev.: **G**lpF, glycerol uptake facilitator or aquaglyceroporin; GlpK, glycerol kinase; GlpD, glycerol-3-phosphate dehydrogenase; Tpi, Triose-phosphate isomerase; Glyc-3-P, glycerol-3-phosphate; DHAP, dihydroxyacetone phosphate; G-3-P, glyceraldehyde-3-phosphate.

## 3. Results and discussion

Table 1 shows the PCR primers used to amplify *glpF* (glycerol uptake facilitator or aquaglyceroporin), *glpK* (glycerol kinase), *glpD* (glycerol 3-phosphate dehydrogenase), and the contiguous *glpFK* genes from *E. coli* K12. These amplicons were spliced using an In-