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

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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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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).

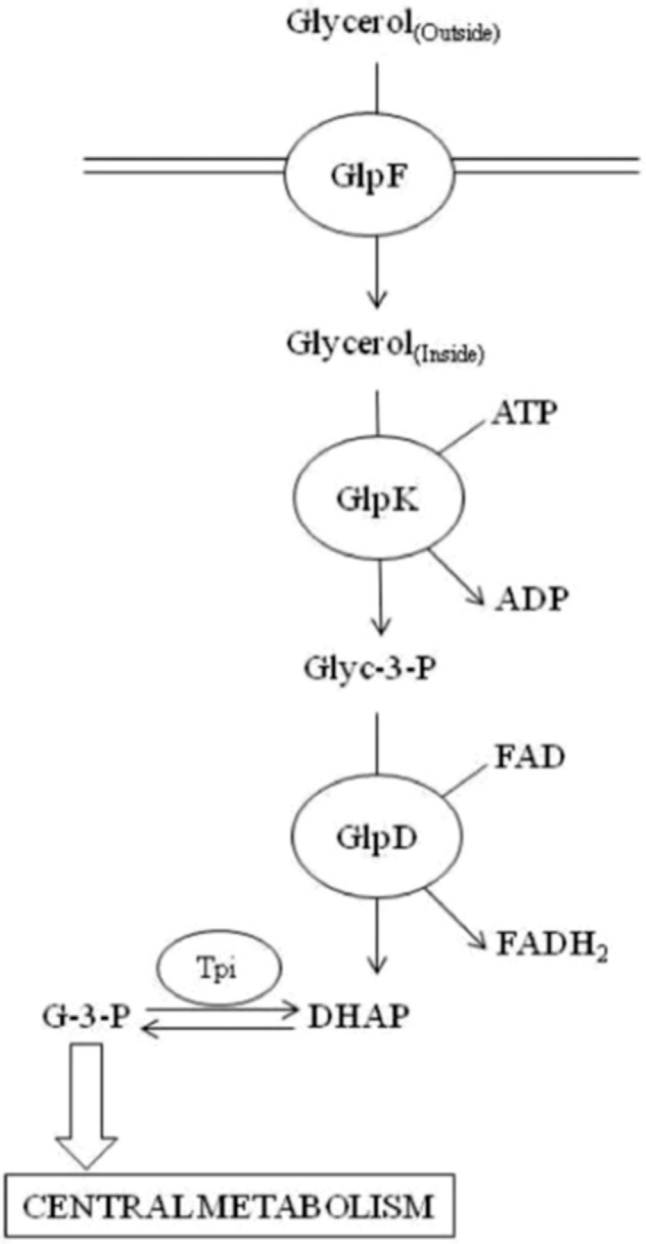

Fig. 1. Schematic of aerobic glycerol assimilation pathway. Abbrev.: **G**lpF, glycerol uptake facilitator or aquaglyceroporin; GlpK, glycerol kinase; GlpD, glycerol-3-phosphate dehydrogenase; Tpi, Triose-phosphate isomerase; Glyc-3-P, glycerol-3-phosphate; DHAP, dihydroxyacetone phosphate; G-3-P, glyceraldehyde-3-phosphate.

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-

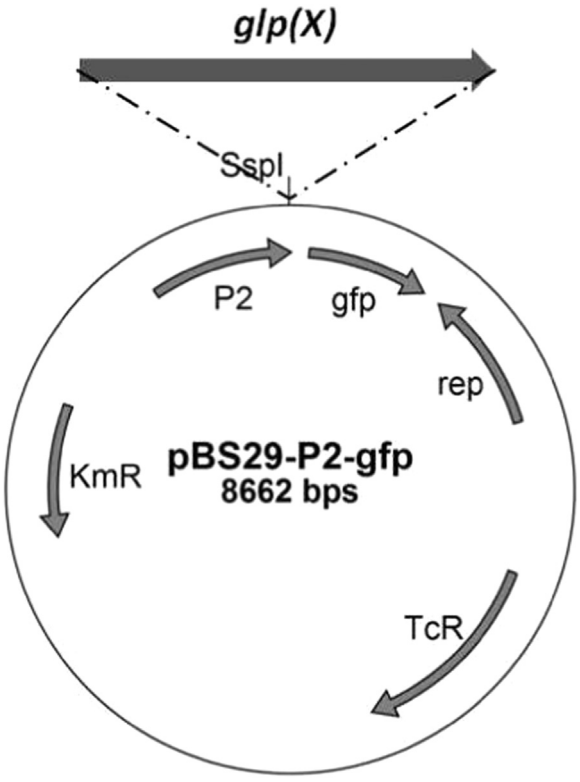

Fig. 2. Schematic of recombinant plasmids pBS29P2-*glp(X)*. Abbrev.: P2, promoter sequence from *P. syringae* (Solaiman and Swingle, 2010); gfp, green-fluorescence-protein; rep, replication region; KmR, kanamycin-resistance gene; TcR, tetracycline-resistance gene; *glp(X)*, the PCR-cloned *E. coli glpF* (glycerol uptake facilitator or aquaglyceroporin), *glpK* (glycerol kinase), *glpD* (glycerol 3-phosphate dehydrogenase), or *glpFK* (the contiguous *glpF* and *glpK* genes).

Fusion cloning technique (Clonetech) into the unique *Ssp*I site of pBS29P2-*gfp*. The recombinant plasmids (Fig. 2) were selected in *E. coli* based on tetracycline resistance or on the absence of green-fluorescence-protein (GFP) fluorescence. The subsequent transformation of *P. chlororaphis* by electroporation with the pBS29P2-*glp(X)* plasmids isolated from *E. coli* resulted in the identification and isolation of genetically engineered *P. chlororaphis* containing pBS29P2-*glpF* (9.6 kb), pBS29P2-*glpK* (10.2 kb), and pBS29P2-*glpFK* (11.1 kb).

We next studied the growth and glycerol consumption of these *P. chlororaphis* transformants in shake-flask experiments. Our preliminary studies showed that *P. chlororaphis* [pBS29P2-*glpFK*], but not *P. chlororaphis* [pBS29P2-*glpK*] and *P. chlororaphis* [pBS29P2-*glpF*], consistently exhibited a growth advantage when glycerol was supplied as the sole carbon source (data not shown). For subsequent experiments, we focused on characterization of *P. chlororaphis* [pBS29P2-*glpFK*] in comparison to the [pBS29P2-*gfp*]- and [pBS29P2-*glpF*]-transformed strains.

The growth of test strains in MSM +0.5% (w/v) glycerol (MSM+$G_{0.5}$) is shown in Fig. 3A. Among the three test strains, *P. chlororaphis* [pBS29P2-*glpFK*] started to grow the earliest after a short lag time (*ca.* 15 h), reaching a stationary state at *ca.* 30 h. *P. chlororaphis* [pBS29P2-*gfp*] control and [pBS29P2-*glpF*] strains grew only after a very long lag phase of 25 h, tapering off at *ca.* 35 h. The difference was most obvious at 25 h, in which *P. chlororaphis* [pBS29P2-*glpFK*] culture had reached an $A_{600\ nm}$ of 2.5, while the growth of [-*gfp*] Control and [-*glpF*] was only beginning with an $A_{600\ nm}$ of *ca.* 1. The glycerol consumption curves (Fig. 3B) show that *P. chlororaphis* [pBS29P2-*glpFK*] started to consume glycerol at a much earlier time than the other two strains, nearly exhausting the glycerol at *ca.* 30 h while the other two cultures still contained *ca.* 40% of the substrate. From these data, it is apparent that *P. chlororaphis* [pBS29P2-*glpFK*] has a cell growth and glycerol utilization advantage compared to the other two strains.

We next assessed the advantageous properties of *P. chlororaphis* [pBS29P2-*glpFK*] in MSM +1.0% (w/v) glycerol (MSM+$G_{1.0}$). The results showed that the *glpFK*-expressing cells started to grow at a much earlier time-point (*ca.* 11 h) than the other two strains (*ca.* 24–27 h), reaching a stationary phase at *ca.* 37 h as opposed to *ca.* 49 h for the control and the *glpF*-expressing strains (Fig. 3C). Fig. 3D shows that at 22 h, half of the initial glycerol had been consumed by *P. chlororaphis* [pBS29P2-*glpFK*]. In contrast, < 20% of the glycerol was consumed by *P. chlororaphis* [pBS29P2-*gfp*] or [pBS29P2-*glpF*] at 22 h. As expected, MSM+$G_{1.0}$ with more substrate afforded a higher cell mass at final $A_{600\ nm}$ values of ~6 (Fig. 3C), while the final $A_{600\ nm}$'s in MSM+$G_{0.5}$ were only ~3 (Fig. 3A).

The long lag time observed in the growth curves of the control-strain *P. chlororaphis* [pBS29P2-*gfp*] reflects the necessity of *P. chlororaphis* to adapt to growth on glycerol perhaps via the derepression of native *glp* genes. Transcriptional regulation of *glp* genes in pseudomonads has been reported (Nikel et al., 2014; Schweizer and Po, 1996) but studies on the glycerol metabolism of *P. chlororaphis*are lacking. Based on the results in Fig. 3, we propose that *P. chlororaphis* wild-type also harbors the *glp* operon under transcriptional regulation. To support this notion, we performed a bioinformatic analysis and found in fact that many *P. chlororaphis* strains contain *glpF*, *glpK*, *glpD* and *glpR* genes or their homologs (data not shown). We further surmised that these genes are also repressed in the wild-type *P. chlororaphis*, leading to the observed long lag time when grown on glycerol (Fig. 3). The constitutive expression of heterologous *glpF* and *glpK* in *P. chlororaphis* [pBS29P2-*glpFK*] thus circumvents the derepression process of the native *glp* genes, leading to a much shortened cell-growth lag time and earlier glycerol consumption (Fig. 3).

Finally, we repeated the fermentation study in MSM+$G_{1.0}$ but under a low aeration condition by using 0.5-l capacity Erlenmeyer flasks which restrict the extent of aeration. Fig. 3E showed that cell growth of all three strains was poor under this condition, tapering off at $A_{600\ nm}$ of only ~3 after 3 days. Glycerol consumption curves (Fig. 3F) showed that all three strains did not completely consume the substrate after the 70-h fermentation; 30–45% of the initial glycerol remained in the culture broth. These results support the notion that glycerol assimilation via GlpFK was an oxidative pathway which required an electron acceptor (in this case oxygen) for the regeneration of FAD needed in the assimilation pathway (Fig. 1) (UniProt Consortium, 2016).

In conclusion, *P. chlororaphis* [pBS29P2-*glpFK*] grows favorably using glycerol as the sole carbon source. The initial lag time of cell growth and the timely assimilation of glycerol substrate are both improved with this transformant. The shorter growth time should result in a favorable techno-economic condition for bioprocesses using glycerol as a substrate..

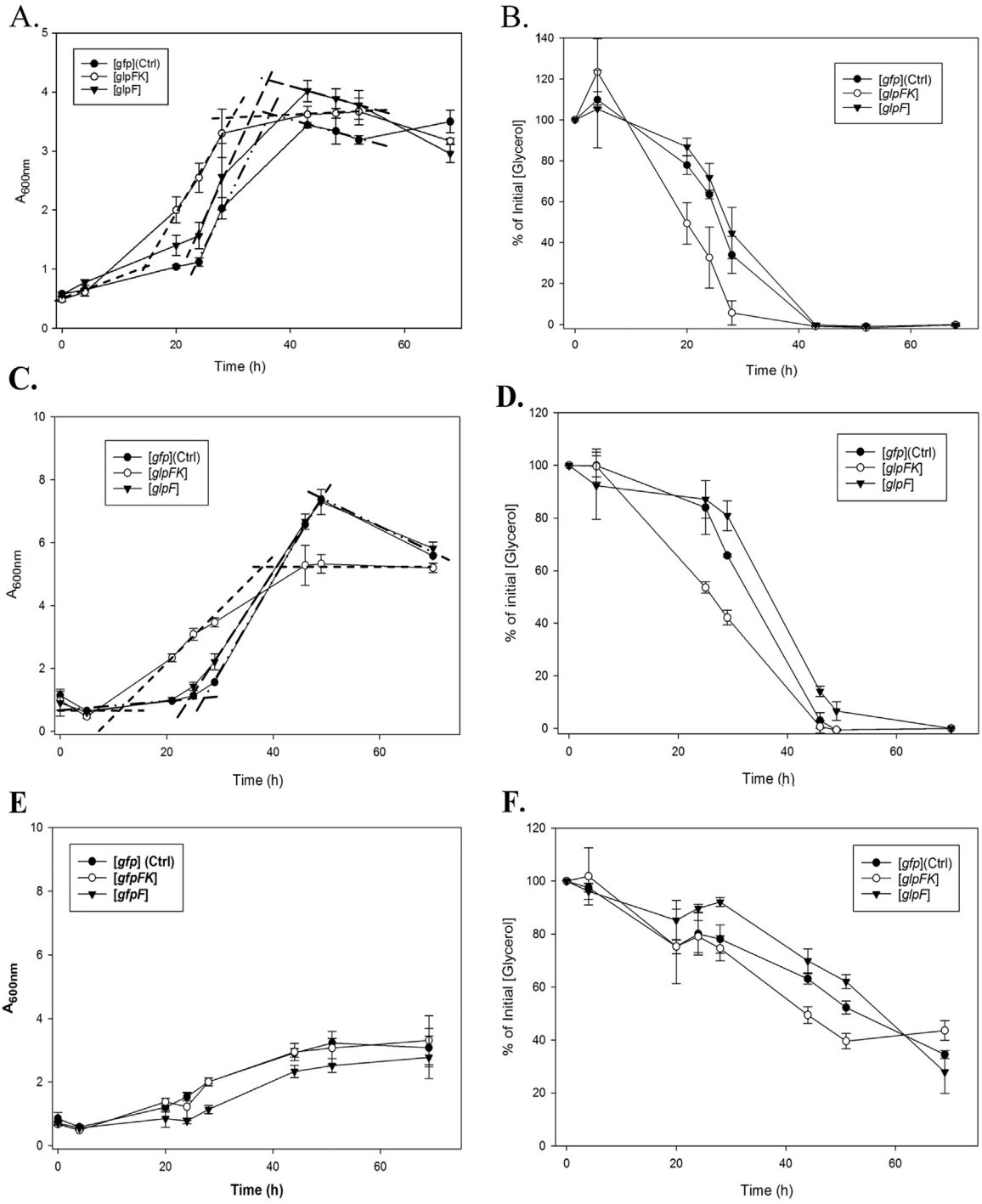

Fig. 3. Shake-flask fermentation in MSM + glycerol media. Culture conditions were 200-ml MSM + 0.5% (**A, B**) – 1.0% (**C-F**) (w/v) Glycerol medium in 2-l (**A-D) or 0.5-l (E-F)** Erlenmeyer flasks incubated at 30 °C with 200-rpm orbital shaking. **A, C, E;** Cell growth monitored by absorbance at 600 nm. **B, D, F;** Glycerol consumption curves. Symbols: (●), Control culture *P. chlororaphis* [pBS29P2-*gfp*]; (○), *P. chlororaphis* [pBS29P2-*glpFK*]; (▾), *P. chlororaphis* [pBS29P2-*glpF*].

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