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Mutational Analyses of Glucose Dehydrogenase and Glucose-6-Phosphate Dehydrogenase Genes in *Pseudomonas fluorescens* Reveal Their Effects on Growth and Alginate Production

Susan Maleki, Mali Mærk, Svein Valla, Helga Ertesvåg

Department of Biotechnology, NTNU Norwegian University of Science and Technology, Trondheim, Norway

The biosynthesis of alginate has been studied extensively due to the importance of this polymer in medicine and industry. Alginate is synthesized from fructose-6-phosphate and thus competes with the central carbon metabolism for this metabolite. The alginate-producing bacterium *Pseudomonas fluorescens* relies on the Entner-Doudoroff and pentose phosphate pathways for glucose metabolism, and these pathways are also important for the metabolism of fructose and glycerol. In the present study, the impact of key carbohydrate metabolism enzymes on growth and alginate synthesis was investigated in *P. fluorescens*. Mutants defective in glucose-6-phosphate dehydrogenase isoenzymes (Zwf-1 and Zwf-2) or glucose dehydrogenase (Gcd) were evaluated using media containing glucose, fructose, or glycerol. Zwf-1 was shown to be the most important glucose-6-phosphate dehydrogenase for catabolism. Both Zwf enzymes preferred NADP as a coenzyme, although NAD was also accepted. Only Zwf-2 was active in the presence of 3 mM ATP, and then only with NADP as a coenzyme, indicating an anabolic role for this isoenzyme. Disruption of *zwf-1* resulted in increased alginate production when glycerol was used as the carbon source, possibly due to decreased flux through the Entner-Doudoroff pathway rendering more fructose-6-phosphate available for alginate biosynthesis. In alginate-producing cells grown on glucose, disruption of *gcd* increased both cell numbers and alginate production levels, while this mutation had no positive effect on growth in a non-alginate-producing strain. A possible explanation is that alginate synthesis might function as a sink for surplus hexose phosphates that could otherwise be detrimental to the cell.

Knowing how carbon sources are channeled through different metabolic pathways to provide the energy and precursors needed to sustain growth is crucial for optimizing an organism for industrial bioproduction purposes. It is known that in pseudomonads, glucose is not channeled to the tricarboxylic acid (TCA) cycle via glycolysis, since these bacteria lack the phosphofructokinase gene. Instead, glucose is metabolized via the Entner-Doudoroff (ED) pathway (see Fig. 1) (1). Previous studies have also indicated strongly that fructose and glycerol are mainly converted to glucose-6-phosphate (G6P) via fructose-1,6-bisphosphate (FBP). G6P is then metabolized through the ED pathway (2).

Pseudomonas fluorescens is a diverse species that occupies many niches in nature (3). Like several other species of this genus, it has the ability to produce the linear polymer alginate. This industrially important, nonrepetitive polysaccharide, containing 1,4-linked α-L-guluronic acid and β-D-mannuronic acid, is currently commercially manufactured from brown algae. Bacterial alginate bioproduction is interesting, however, especially because bacteria may be engineered to produce alginates with properties that are well suited for medical applications. By using a nonpathogenic wild-type strain of *P. fluorescens*, we have been able to obtain mutant strains, for example, strain Pf201, with industrial-scale alginate production levels (4).

Alginate is synthesized from fructose-6-phosphate (F6P) and thus competes with the central carbon metabolism for FBP. This motivated us to investigate how carbon flow through the various early pathways of the central carbohydrate metabolism would influence cell growth and alginate yield. Figure 1 shows the different metabolic pathways for the degradation of glucose, fructose, and glycerol in *P. fluorescens*. We wanted to study the contributions of glucose dehydrogenase (Gcd) and glucose-6-phosphate dehydrogenase (G6PDH; also called Zwf) to these processes. Lessmann et al. (5) have described two different G6PDHs from *P. fluorescens* and have shown that the activity of one of these isoenzymes did not vary much with the carbon sources tested, while the other was induced by glucose (5). It is not clear, however, whether the two *P. fluorescens* Zwf enzymes have strictly defined roles, one being catabolic and the other anabolic, or if either one would be sufficient for growth. The *P. fluorescens zwf-1* gene is the first gene in an operon that also contains *pgl* (encoding 6-phosphogluconolactonase) and *eda* (encoding 2-keto-3-deoxy-6-phosphogluconate [KDPG] aldolase), while *zwf-2* is found downstream of the 6-phosphogluconate (6PGA) dehydrogenase gene (*gnd*) in another operon. These differing genomic contexts indicate that Zwf-1 functions primarily in the ED pathway, while Zwf-2 is more important in the pentose phosphate (PP) pathway (Fig. 1). Lessmann et al. (5) proposed that the Zwf enzyme linked to the ED pathway was the one that was inhibited by ATP, since this would enable metabolic control of the enzyme (6, 7). They also found that the enzymes dif-

Received 4 November 2014 **Accepted** 1 March 2015

Accepted manuscript posted online 6 March 2015

Citation Maleki S, Mærk M, Valla S, Ertesvåg H. 2015. Mutational analyses of glucose dehydrogenase and glucose-6-phosphate dehydrogenase genes in *Pseudomonas fluorescens* reveal their effects on growth and alginate production. Appl Environ Microbiol 81:3349–3356. doi:10.1128/AEM.03653-14.

Editor: H. Nojiri

Address correspondence to Helga Ertesvåg, helga.ertesvag@ntnu.no.

M.M. and S.M. contributed equally to this article.

doi:10.1128/AEM.03653-14

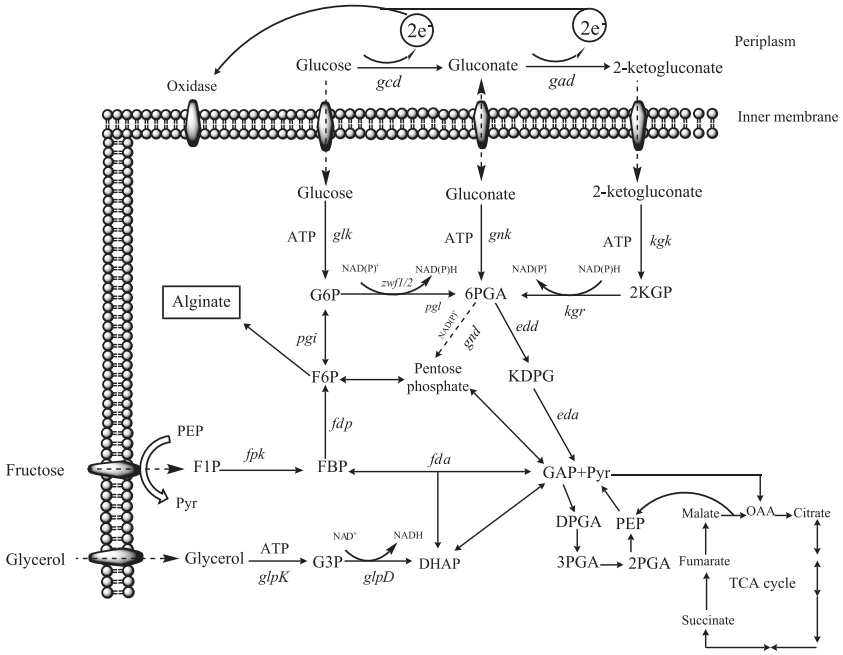

FIG 1 Uptake and utilization of glucose, fructose, and glycerol in *P. fluorescens*. Abbreviations: G6P, glucose-6-phosphate; 6PGA, 6-phosphogluconate; 2KGP, 2-keto-6-phosphogluconate; KDPG, 2-keto-3-deoxy-6-phosphogluconate; F6P, fructose-6-phosphate; FBP, fructose-1,6-bisphosphate; F1P, fructose-1-phosphate; GAP, glyceraldehyde-3-phosphate; Pyr, pyruvate; DPGA, 1,3-diphosphoglycerate; 3PGA, 3-phosphoglycerate; 2PGA, 2-phosphoglycerate; PEP, phosphoenolpyruvate; OAA, oxaloacetate; G3P, glycerol-3-phosphate; DHAP, dihydroxyacetone phosphate; Gad, gluconate dehydrogenase; Gcd, glucose dehydrogenase; Kgk, 2-ketogluconate kinase; Gnk, gluconokinase; Glk, glucokinase; Kgr, 2KGP reductase; Gnd, 6PGA dehydrogenase; GlpK, glycerol kinase; GlpD, G3P dehydrogenase; Zwf1/2, G6P dehydrogenases; Pgi, phosphoglucose isomerase; Pgl, 6-phosphogluconolactonase; Edd, 6PGA dehydratase; Eda, KDPG aldolase; Fpk, 1-phosphofructokinase; Fdp, fructose-1,6-bisphosphatase; Fda, fructose-1,6-bisphosphate aldolase.

fered with regard to cofactor preference (NAD^+ or $NADP^+$). A more thorough understanding of these enzymes and the extent to which their functions in the cell are overlapping is needed for correct modeling of the metabolism of the cell.

In most studies on the carbon flow in *Pseudomonas* spp., glucose has been used as the carbon source. However, for industrial bioproduction purposes, cheaper carbon sources, such as glycerol, are more attractive, because they could make the cost of microbial alginates more competitive, leading to a broader potential range of applications. It is not common to modify the central carbohydrate metabolism for bioprocess development, but since alginate is made from F6P, we hypothesized that inactivation of key genes such as *gcd*, *zwf-1*, or *zwf-2* might lead to stimulation of alginate biosynthesis. To make the study directly relevant for bioprocess development, we used strain Pf201 as a starting point. Our results show that although alginate synthesis in this strain is generally not very sensitive to the gene inactivations investigated, disruption of *zwf-1* nevertheless has a very clear stimulatory effect on polymer synthesis when glycerol is used as the carbon source.

MATERIALS AND METHODS

Strains, media, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *P. fluorescens* and *Escherichia coli* strains were routinely grown in L broth (10 g/liter tryptone, 5 g/liter yeast extract, 5 g/liter NaCl) in 250-ml shake flasks at 225 rpm at 30 and 37°C, respectively. Conjugations between *E. coli* and *P. fluorescens* strains were carried out on L agar (L broth containing 15 g/liter agar), and transconjugants were selected on *Pseudomonas* isolation agar (PIA; Difco) with appropriate antibiotics added. For growth experiments, alginate production, and G6PDH activity assays, 1% LB-grown precultures were inoculated in DEF3 minimal medium, consisting of 0.39 g/liter yeast extract, 0.65 g/liter KH_2HPO_4, 2.73 g/liter $(NH_4)_2HPO_4$, 0.9 g/liter citric acid·H_2O, 0.02 g/liter ferric citrate, 0.001 g/liter H_3BO_3, 0.005 g/liter $MnCl_2{\cdot}4H_2O$, 0.0039 g/liter EDTA·$2H_2O$, 0.0005 g/liter $CuCl_2{\cdot}2H_2O$, 0.0008 g/liter $Na_2Mo_4O_4{\cdot}2H_2O$, 0.0008 g/liter $CoCl_2{\cdot}6H_2O$, 0.0027 g/liter $Zn(CH_3COO)_2{\cdot}2H_2O$, 1.56 g/liter NaCl, 0.57 g/liter $MgSO_4{\cdot}7H_2O$, 5 g/liter morpholinepropanesulfonic acid (MOPS), and 20 g/liter of a carbon source (glucose, fructose, or glycerol). The pH in the medium was adjusted to 7.0 with NaOH. When appropriate, antibiotics were added at the following concentrations: triclosan (Tric), 25 μg/ml; apramycin (Am), 50 μg/ml; ampicillin (Ap), 200 μg/ml; kanamycin (Km), 50 μg/ml; tetracycline (Tc), 15 μg/ml. Proteases (0.15 ml/liter of Alcalase 2.4 L and Neutrase 0.5 L) were added to alginate production media in order to degrade alginate lyases. The *Pm* promoter was induced by the addition of 0.25 mM *m*-toluate. For liquid cultures, an inducer was added 8 h after inoculation. For the detection of LacZ activity, 60 μl of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) solution (20 mg/ml in dimethyl sulfoxide [DMSO]) was added to each agar plate 30 min before the plating of bacteria.

DNA standard techniques. All routine DNA manipulation methods, transformation, and cloning were performed as described previously (8). *E. coli* strains S17.1 and S17.1 λ*pir* were used as hosts for standard cloning procedures and the replication of suicide plasmids. The sequences of all amplified DNA fragments were confirmed by DNA sequencing.

Construction of strains. To generate *P. fluorescens* Δ*zwf-1*, Δ*zwf-2*, and Δ*gcd* mutants, homologous recombination procedures were performed using pMG48 derivatives (pMM7, pMM6, and pMM5, respectively) as described previously (9) (see Table 1 for details on cloning). All mutant strains constructed were verified by PCR and restriction digestion.

Genes were overexpressed by constructing transposon vectors where the genes in question were placed under the control of the inducible

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
E. coli		
S17.1	RP4::2-Tc::Mu-Km::Tn*7 pro res mod*$^+$	22
S17.1 λ*pir*	λ*pir recA thi pro hsdR* M$^+$ RP4::2-Tc::Mu-Km::Tn*7* Tp^r Sm^r	23
P. fluorescens		
NCIMB 10525	Nonmucoid wild type; Tric^r	NCIMB
NCIMB 10525 Δ*zwf-1*	*zwf-1* in-frame deletion mutant of NCIMB 10525; Tric^r	This study
NCIMB 10525 Δ*zwf-2*	*zwf-2* in-frame deletion mutant of NCIMB 10525; Tric^r	This study
NCIMB 10525 Δ*gcd*	*gcd* in-frame deletion mutant of NCIMB 10525; Tric^r	This study
NCIMB 10525 Δ*zwf-1* Δ*zwf-2*	*zwf-2* in-frame deletion mutant of NCIMB 10525 Δ*zwf-1*; Tric^r	This study
NCIMB 10525 Δ*zwf-1* Δ*gcd*	*gcd* in-frame deletion mutant of NCIMB 10525 Δ*zwf-1*; Tric^r	This study
NCIMB 10525 Δ*zwf-2* Δ*gcd*	*zwf-2* in-frame deletion mutant of NCIMB 10525 Δ*gcd*; Tric^r	This study
NCIMB 10525 Δ*zwf-1*::TnSM1	Derivative of NCIMB 10525 Δ*zwf-1* in which *zwf-2* is overexpressed by inserting the transposon from pSM1; Am^r Tric^r	This study
NCIMB 10525 Δ*zwf-2*::TnMM12	Derivative of NCIMB 10525 Δ*zwf-2* in which *zwf-1* is overexpressed by inserting the transposon from pMM12; Km^r Tric^r	This study
NCIMB 10525 Δ*zwf-1* Δ*zwf-2*::TnMM12	Derivative of NCIMB 10525 Δ*zwf-1* Δ*zwf-2* in which *zwf-1* is overexpressed by inserting the transposon from pMM12; Km^r Tric^r	This study
NCIMB 10525 Δ*zwf-1* Δ*zwf-2*::TnSM1	Derivative of NCIMB 10525 Δ*zwf-1* Δ*zwf-2* in which *zwf-2* is overexpressed by inserting the transposon from pSM1; Am^r Tric^r	This study
NCIMB 10525 Δ*zwf-1* Δ*gcd-2*::TnHE418	Derivative of NCIMB 10525 Δ*zwf-1* Δ*gcd* in which *gcd* is overexpressed by inserting the transposon from pHE418; Am^r Tric^r	This study
Pf201	Mucoid mutant derived from NCIMB 10525	4
Pf201 Δ*zwf-1*	Nonmucoid *zwf-1* in-frame deletion mutant of Pf201; Tric^r	This study
Pf201 Δ*zwf-2*	*zwf-2* in-frame deletion mutant of Pf201; Tric^r	This study
Pf201 Δ*gcd*	*gcd* in-frame deletion mutant of Pf201; Tric^r	This study
Pf201 Δ*zwf-1* Δ*gcd-2*::TnHE418	Derivative of Pf201 Δ*zwf-1* Δ*gcd* in which *gcd* is overexpressed by inserting the transposon from pHE418; Am^r Tric^r	This study
Pf201 Δ*zwf-1* MM27	Derivative of Pf201 Δ*zwf-1* where wild-type *zwf-1* was restored by gene exchange using pMM27; Tric^r	This study
Pf201 Δ*algC*::TnKB60	Pf201 Δ*algC* with a transposon insertion from pKB60 (containing *algC* under *Pm-G5* control); Km^r Tric^r	8
Pf201 Δ*algC* Δ*zwf-1*::TnKB60	*zwf-1* in-frame deletion mutant of Pf201 Δ*algC*::TnKB60; Km^r Tric^r	This study
Pf201 Δ*algC*::TnKB60 MM27	Derivative of Pf201 Δ*algC* Δ*zwf-1*::TnKB60 where wild-type *zwf-1* was restored by gene exchange using pMM27; Km^r Tric^r	This study
Plasmids		
pHKK-phOx-scFv-c-MYC-His6	pHKK with *phOx-scFv-c-myc* inserted between *pelB* and the His_6 coding sequence; Ap^r	24
pKD20	Transposon delivery vector; Ap^r Km^r	8
pMG48	RK2-based suicide gene replacement vector; Ap^r Tc^r	9
pEM1	Derivative of pLitmus28 into which a 1.2-kb EcoRI-NcoI DNA fragment from pKD20 containing the *Pm-G5* promoter derivative was inserted; Ap^r	This study
pYQ1	Derivative of pKD20 where the kanamycin resistance gene is replaced by a gene encoding apramycin resistance; Am^r	This study
pKB64	Derivative of pKD20 where the kanamycin resistance gene is replaced by *tetA-tetR*, encoding tetracycline resistance; Tc^r	This study
pMM1	Derivative of pHKK-phOx-scFv-c-MYC-His6 in which a 2.6-kb NdeI-NotI-digested PCR-fragment containing *P. fluorescens gcd* was inserted; Ap^r	This study
pMM2	Derivative of pHKK-phOx-scFv-c-MYC-His6 in which a 1.6-kb NdeI-NotI-digested PCR-fragment containing *P. fluorescens zwf-2* was inserted; Ap^r	This study
pMM5	Derivative of pMM1 where a 0.4-kb DNA fragment was removed using PciI, introducing an in-frame deletion (designated Δ*gcd*) into *gcd*; Ap^r	This study
pMM6	Derivative of pMM2 where a 0.09-kb DNA fragment was deleted using BstBI, introducing an in-frame deletion (designated Δ*zwf-2*) into *zwf-2*; Ap^r	This study
pMM7	Derivative of pMM11 where a 0.4-kb DNA fragment was removed by OliI, introducing an in-frame deletion (designated Δ*zwf-1*) into *zwf-1*; Ap^r	This study
pMM8	Derivative of SpeI-NotI-restricted pMG48 into which a 1.5-kb XbaI-NotI DNA fragment from pMM6 containing Δ*zwf-2* was inserted; Ap^r Tc^r	This study
pMM9	Derivative of SpeI-NotI-restricted pMG48 into which Δ*gcd* from pMM5 (XbaI-NotI) was inserted; Ap^r Tc^r	This study
pMM10	Derivative of SpeI-NotI-restricted pMG48 into which Δ*zwf-1* from pMM7 (XbaI-NotI) was inserted; Ap^r Tc^r	This study
pMM11	Derivative of pEM1 into which a 1.5-kb NdeI-NotI-digested PCR fragment containing *P. fluorescens zwf-1* was inserted; Ap^r	This study
pMM12	Derivative of pKD20 into which an NdeI-NotI-digested DNA fragment carrying *zwf-1* from pMM11 was inserted so that *zwf-1* is controlled by *Pm-G5*; Ap^r Km^r	This study
pMM13	Derivative of NdeI-NotI-restricted pKB64 into which *zwf-1* from pMM11 (NdeI-NotI) was inserted so that *zwf-1* is controlled by *Pm-G5*; Ap^r Tc^r	This study
pMM27	Derivative of SpeI-NotI-restricted pMG48 into which *zwf-1* from pMM11 (XbaI-NotI) was inserted; Ap^r Tc^r	This study
pSM1	Derivative of pYQ1 into which a 1.5-kb NdeI-NotI-digested DNA fragment from pMM2 carrying *zwf-2* was inserted so that *zwf-2* is controlled by *Pm-G5*; Am^r	This study
pHE418	Derivative of pYQ1 into which a 2.6-kb NdeI-NotI-digested DNA fragment from pMM1 carrying *gcd* was inserted so that *gcd* is controlled by *Pm-G5*; Am^r	This study

Pm-G5 promoter (4). These plasmids were then transferred to the relevant strains by conjugation, and transposon insertion mutants were selected using Km or Am. Several different transposon insertion mutants were evaluated in order to avoid choosing mutant strains in which the phenotype observed was caused by the transposon insertion site and not by induced gene expression.

Alginate quantification. *P. fluorescens* cell culture samples were harvested, diluted using 0.2 M NaCl to decrease viscosity when necessary, and

centrifuged at 13,000 rpm for 10 min to remove cells. The alginate in the supernatants was deacetylated by a mild alkaline treatment as described previously (10). Alginate was quantified using M-specific lyase (AlxM; *Pseudomonas* sp.) and G-specific lyase (AlyA; *Klebsiella aerogenes*) as described previously (11). All alginate quantifications were performed in triplicate.

G6PDH activity assays. Cell extracts containing G6PDH were prepared from overnight cultures of *P. fluorescens* NCIMB 10525 Δ*zwf-2*::TnMM12 and *P. fluorescens* NCIMB 10525 Δ*zwf-1*::TnSM1 using B-PER II bacterial protein extraction reagent (Pierce). Cell extracts were stored at −80°C. Total protein concentrations in the samples were estimated using a colorimetric protein assay (Bio-Rad) based on the Bradford method (12). Bovine serum albumin was used to generate the standard curve.

G6PDH activity was determined by measuring $NAD(P)^+$ reduction spectrophotometrically at 340 nm. The enzyme assay was performed according to the protocol described previously by Lynch et al. (13) but modified for measurements in 96-well UV plates. Ten microliters of the cell extract and 190 μl of the assay solution were mixed to start the reaction. In order to investigate the inhibitory effect of ATP on the enzymes, activity was assayed in the presence and absence of 3 mM ATP. Assay mixtures without G6P were used to measure nonspecific reduction of $NAD(P)^+$. A_{340} was measured every 10 s for 60 min, and values were converted to micromolar concentrations of NAD(P)H, i.e., reduced $NAD(P)^+$ (6). One unit of enzyme is defined as the amount of total soluble protein that catalyzes the reduction of 1 μmol of $NAD(P)^+$ per min.

RESULTS

Properties of the two *P. fluorescens* G6PDH isoenzymes. G6PDH (encoded by *zwf*) is required in both the ED and PP pathways. Knowledge of the properties of the two Zwf isoenzymes is therefore important for understanding the carbohydrate metabolism of *P. fluorescens*. The *zwf-1* and *zwf-2* genes were first separately inactivated by in-frame deletions in the wild-type *P. fluorescens* strain NCIMB 10525, and then a Δ*zwf-1* Δ*zwf-2* double mutant was constructed. Cell extracts from the single mutant strains, especially NCIMB 10525 Δ*zwf-1*, showed low levels of G6PDH activity. As expected, no G6PDH activity was detected in cell extracts from the double mutant. In order to analyze the properties of each enzyme separately *in vitro*, each functional gene was cloned into a transposon and was placed under the control of the inducible *Pm*-G5 promoter. The transposons were transferred to the respective deletion strains, generating strains NCIMB 10525 Δ*zwf-2*::TnMM12 (expressing only *zwf-1*) and NCIMB 10525 Δ*zwf-1*::TnSM1 (expressing only *zwf-2*). Extracts from these strains were then used to analyze the properties of each enzyme separately. The strains were grown in LB overnight, and a 1% inoculum was transferred to DEF3 minimal medium containing glucose as the carbon source. The cultures were grown for 8 h before *Pm*-G5 was induced by the addition of *m*-toluate, and cultivation was continued overnight. G6PDH activity was measured in cell extracts from these strains as described in Materials and Methods. Both NAD^+ and $NADP^+$ were used as coenzymes, in separate experiments.

The results showed that both enzymes display the highest activity with $NADP^+$ as the coenzyme, but the difference was much smaller for Zwf-1 than for Zwf-2 (Fig. 2). Furthermore, addition of 3 mM ATP resulted in no detectable Zwf-1 activity with either coenzyme. Zwf-2 also lost its activity when assayed with NAD^+ and ATP but retained 60% activity in the presence of ATP when $NADP^+$ was used as the coenzyme. These data indicate that Zwf-2 is active even when the energy status of the cell is good, probably taking part in the pentose phosphate pathway operating in the anabolic direction. Lessmann et al. (5) reported that the most highly expressed isoenzyme, now annotated as Zwf-1, showed a slight preference for NAD^+ (5), while our results indicate that both enzymes prefer $NADP^+$ to NAD^+ as a cofactor. The reasons for this discrepancy are not known but could, for instance, be due to strain-dependent differences.

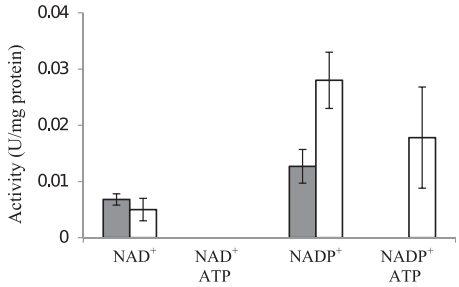

FIG 2 Coenzyme preference and ATP-induced inhibition of the glucose-6-phosphate dehydrogenases Zwf-1 (shaded bars) and Zwf-2 (open bars). Enzyme activities are given as average values measured in cell extracts obtained from three independently grown cultures of strains expressing only one of the two G6PDH isoenzymes. Extracts were assayed using NAD^+ or $NADP^+$ as the coenzyme, and with or without the addition of ATP. (See Materials and Methods for details.) One unit of enzyme is defined as the amount of total soluble protein that catalyzes the reduction of 1 μmol $NAD(P)^+$ per min.

The phenotypes of *gcd*, *zwf-1*, and *zwf-2* mutants with respect to growth depend on the carbon source. In *P. fluorescens*, glucose is channeled into the central carbohydrate metabolism either via glucose dehydrogenase (Gcd) or via glucokinase (Glk) and G6PDH/Zwf (1). Both pathways converge in the common product KDPG (Fig. 1). It has been shown that this metabolite binds to and releases the repressor HexR from promoters controlling the expression of Zwf-1 and other key enzymes in the ED pathway (14). We wanted to investigate the effect of inactivating the three genes *gcd*, *zwf-1*, and *zwf-2* by assessing the impact on growth when glucose, fructose, or glycerol was used as the carbon source. Since alginate production imposes a drain on F6P that can also affect growth (15), these mutants were generated from the non-alginate-producing wild-type strain NCIMB 10525. Strains carrying single and double mutations were constructed.

Growth curves for wild-type and mutant strains cultivated in minimal medium with glucose as the carbon source are shown in Fig. 3a. The three single gene deletion strains displayed different growth kinetics. The Δ*zwf-2* strain grew very similarly to the wild type, while the Δ*gcd* strain grew slightly slower. The Δ*zwf-1* mutant displayed a prolonged lag-phase but nevertheless reached a much higher final optical density (OD) than all the other strains. The growth phenotypes of the Δ*zwf-1* Δ*zwf-2* and Δ*zwf-2* Δ*gcd* double mutants indicated moderate additive negative effects of combining the two deletions. The most drastic negative effect on growth was observed for the strain where both *gcd* and *zwf-1* were inactivated. As a control, TnHE418, where *gcd* is controlled by the inducible *Pm*-G5 promoter, was transferred to strain NCIMB 10525 Δ*zwf-1* Δ*gcd*. As expected, the complemented strain grew similarly to strain NCIMB 10525 Δ*zwf-1* (results not shown). Our results demonstrate that the activity of Zwf-2 alone is not sufficient to sustain normal growth rates on glucose and suggest that most of the carbon flow from glucose is channeled through the reactions catalyzed by the *gcd* and *zwf-1* gene products.

In cultivations on fructose (Fig. 3b) the Δ*zwf-1* mutant displayed a strongly reduced growth rate and did not recover at late

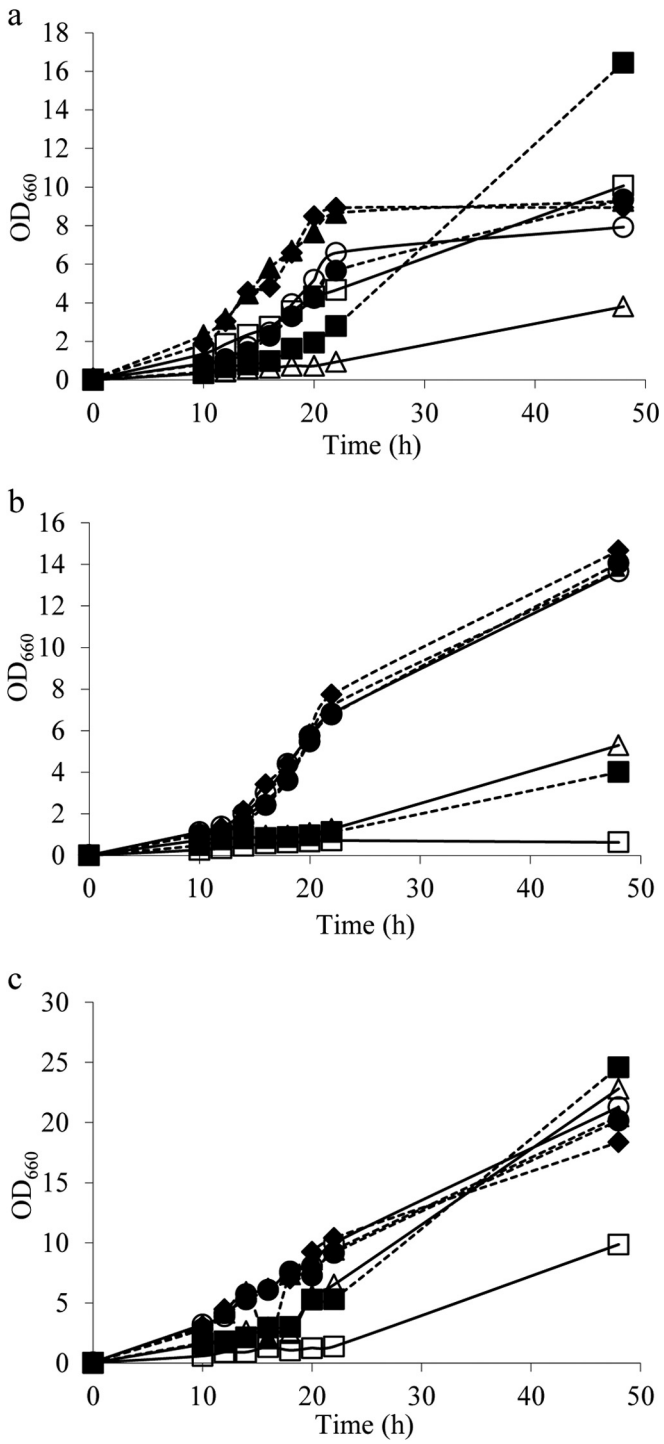

FIG 3 Growth of *P. fluorescens* NCIMB 10525 and its mutant derivatives on various carbon sources. The strains were cultivated in minimal medium using glucose (a), fructose (b), or glycerol (c) as the carbon source, and growth was measured as the absorbance at 660 nm. The following strains were investigated: *P. fluorescens* NCIMB 10525 (◆) and its Δ*zwf-1* (■), Δ*zwf-2* (▲), Δ*gcd* (●), Δ*zwf-1* Δ*zwf-2* (□), Δ*zwf-1* Δ*gcd* (△), and Δ*zwf-2* Δ*gcd* (○) mutants. Each cultivation was repeated at least three times, each time resulting in growth profiles essentially the same as those displayed here.

stages of growth, as it did on glucose. This difference may occur because glucose, but not fructose, can be metabolized by Gcd. As a consequence, inactivation of *gcd* has no additional negative growth effect on a Δ*zwf-1* mutant when grown on fructose. The Δ*gcd* and Δ*zwf-2* single mutants, as well as the Δ*zwf-2* Δ*gcd* double mutant, grew similarly to the wild type. In contrast, the Δ*zwf-1* Δ*zwf-2* double mutant hardly grew at all on fructose. This indicates that a carbon flow through the reactions catalyzed by the Zwf enzymes, and mostly by Zwf-1, is necessary for the cell under these conditions. This result was unexpected, since fructose enters the central carbon metabolism as fructose-1,6-bisphosphate in *P. fluorescens*, and the bacterium expresses a functional fructose-1,6-biphosphatase (Fig. 1).

As a control, transposons TnMM12 (expressing *zwf-1*) and TnSM1 (expressing *zwf-2*) were transferred separately to the Δ*zwf-1* Δ*zwf-2* double mutant. The phenotypes of the new strains were similar to those of the *zwf-2* and *zwf*-1 single mutants, respectively, confirming that the inability of the double mutant to grow on fructose was caused by the absence of G6PDH enzymes (data not shown). These data also demonstrate that the in frame *zwf-1* deletion introduced by using pMM10 does not have a strong (if any) polar effect on the downstream 6-phosphogluconolactonase gene (*pgl*). The genome contains only one copy of this gene, and Pgl activity is necessary to convert the G6PDH product to 6-phosphogluconate (Fig. 1).

When the strains were grown on glycerol, the only strain exhibiting a severely reduced growth rate was the Δ*zwf-1* Δ*zwf-2* double mutant (Fig. 3c), demonstrating that G6PDH activity is important even when carbon enters the cells as a 3-carbon compound, although it is less important than for fructose-grown cells.

Effects of *zwf-2* and *gcd* inactivation on growth and alginate production. *P. fluorescens* strain Pf201 is a stable high-level alginate-producing mutant (8). We decided to use this strain to study how the channeling of carbon through the different metabolic pathways shown in Fig. 1 affects alginate production. Pf201 mutants with in-frame deletions in *zwf-2* or *gcd* were easily obtained and formed mucoid colonies on PIA, showing that the mutants had retained the ability to produce alginate. The same strategy was applied for *zwf-1*, but unsuccessfully, so for this gene, a more complicated procedure was needed (see below). The Δ*zwf-2* and Δ*gcd* strains were cultivated in defined medium containing glucose, fructose, or glycerol in order to assess growth and alginate production. Since alginate production sequesters carbon and imposes a drain on some of the F6P that could otherwise have been used for generating biomass, the biomass reached was, as expected, lower than that for the isogenic non-alginate-producing strains. Neither of the mutations affected alginate production significantly when the strains were grown on glycerol or fructose (results not shown).

On glucose, the Δ*gcd* mutant produced significantly more alginate than Pf201, while inactivation of *zwf-2* resulted in slightly increased alginate production (Fig. 4). The apparent cell density of Pf201 Δ*gcd* was also higher than that for the other strains (Fig. 4a), but this was not reflected in a higher number of CFU after 24 h (Fig. 4c), indicating that Pf201 Δ*gcd* cultures contain something aside from cell mass that contributes to their high OD at 660 nm (OD_{660}). In order to confirm the phenotype of Pf201 Δ*gcd*, the strain was complemented by transposon TnHE418, carrying *gcd* under the control of the *Pm-G5* promoter. As expected, the complemented strain showed lower OD values and produced less alg-

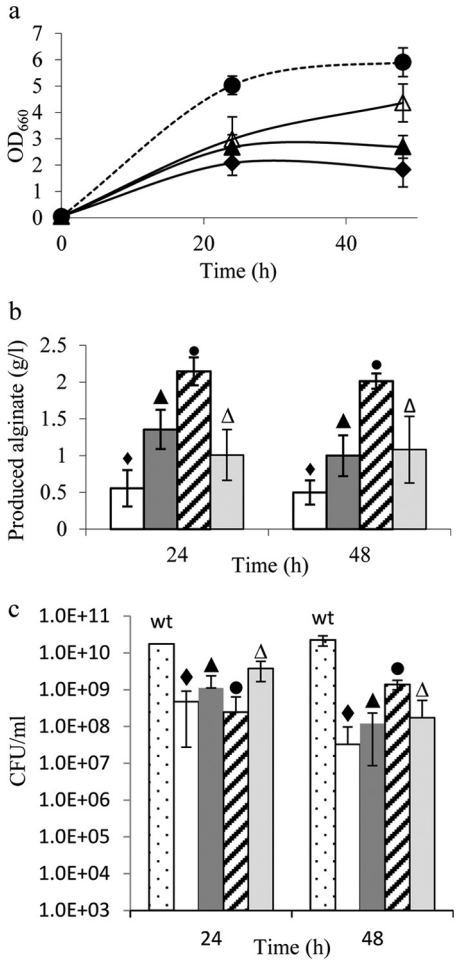

FIG 4 Effects of *zwf-2* and *gcd* inactivation on growth and alginate production in cells cultivated with glucose as the carbon source. (a) Growth, expressed as OD_{660}. Data are averages for three biological replicates, and error bars indicate standard deviations. (b) Alginate production, expressed as grams per liter of culture. Data are averages for three biological replicates. (c) Growth, expressed as CFU per milliliter of culture. Data are averages for at least two biological replicates. Strains: Pf201 (◆, open bars), Pf201 Δ*zwf-2* (▲, dark shaded bars), Pf201 Δ*gcd* (●, striped bars), Pf201 Δ*gcd*::TnHE418 (△, light shaded bars), and the non-alginate-producing wild-type (wt) strain NCIMB 10525 (stippled bars).

inate than Pf201 Δ*gcd*, proving that lack of Gcd was indeed causing the phenotype observed.

A combination of alginate and gluconate production induces cell death. While it is not unusual for the number of viable cells to decrease somewhat in late-stationary phase, the CFU counts for NCIMB 10525, Pf201, and derivatives of Pf201 indicated that when cultivated on minimal medium with glucose as the carbon source, Pf201 exhibited clear signs of extreme stress (Fig. 4c), and in two of three cultures, <1,000 viable cells were present after 48 h. The third culture also displayed a decreased CFU count. No comparable decreases in CFU were detected in cultures cultivated on carbon sources other than glucose (not shown). The CFU count of strain Pf201 Δ*gcd* increased slightly from 24 to 48 h, while the strain complemented with TnHE418 behaved similarly to Pf201. This strongly indicates that simultaneous production of gluconate and alginate resulted in a stress situation that the cells had difficulty coping with, while they were able to thrive when challenged with the production of only one of the acids.

Inactivation of *zwf-1* increases alginate production when glycerol is used as the carbon source. As mentioned above, the frequency of recombinant mutants with an inactivated *zwf-1* gene was found to be much lower in Pf201 than in the wild-type non-alginate-producing strain. Furthermore, all Pf201 Δ*zwf-1* mutants obtained formed nonmucoid colonies on PIA, indicating a deficiency in alginate biosynthesis. Heterologous recombination was used to replace the Δ*zwf-1* gene in one of these mutants with wild-type *zwf-1*, using plasmid pMM27. Even though the genotype of *zwf-1* was confirmed to be restored to wild type by PCR, alginate production was not recovered. We therefore considered it likely that the original mutant strain had acquired a secondary mutation that suppresses alginate synthesis. This would indicate that a lack of Zwf-1 during alginate synthesis imposes a selective growth disadvantage that makes it difficult to obtain *zwf-1* mutants from an alginate-producing strain. A similar problem had been encountered earlier in studying the effect of AlgL on alginate production and was then solved by first constructing a Pf201 derivative with *m*-toluate-inducible *algC* (required for alginate biosynthesis) inserted on a transposon in a Δ*algC* background. An in-frame deletion in *algL* could then be constructed under noninducing conditions (8). Correspondingly, we constructed strain Pf201 Δ*algC* Δ*zwf-1*::TnKB60 (see Table 1 for details). We further used plasmid pMM27 to repair the deletion in *zwf-1*, and the resulting strain (Pf201 Δ*algC*::TnKB60 MM27) was used as an additional control strain. Both these strains were mucoid on PIA in the presence of *m*-toluate, demonstrating that a *zwf-1* deletion can be made with no loss of alginate synthesis.

The strains were cultivated using different carbon sources, and growth and alginate production were monitored (Fig. 5). On glucose, both the growth rate (Fig. 5a) and the alginate production (Fig. 5b) of the strain lacking *zwf-1* (Pf201 Δ*algC* Δ*zwf-1*::TnKB60) were similar to those observed for the control strains (Pf201 Δ*algC*::TnKB60 [not shown], Pf201, and Pf201 Δ*algC* Δ*zwf-1*::TnKB60 MM27).

As expected from the experiments using the wild-type non-alginate-producing strain, the growth rate on fructose was lower for the Pf201 Δ*zwf-1* strain than for Pf201 or the repaired strain containing wild-type *zwf-1* (Fig. 5c). The amounts of alginate produced per unit of culture volume were similar for all three strains (Fig. 5d), but per OD unit, production was somewhat higher for the Δ*zwf-1* strain. Note that the amount of alginate produced with fructose as the carbon source is much higher than that with glucose, as is also the case for the original Pf201 strain.

For glycerol-grown cultures, the initial growth rates were similar for all three strains, but the final cell mass was lower for the Δ*zwf-1* strain (Fig. 5e). Interestingly, this strain still produced significantly more alginate than the two control strains (Fig. 5f). The polymer production per unit of cell mass is therefore even higher. This observation is potentially of biotechnological significance, as discussed below.

DISCUSSION

The results of this study show that among the genes evaluated, inactivation of the G6PDH gene *zwf-1* led to the most pronounced phenotypes with the carbon sources tested. Our *in vitro* data show that Zwf-2 probably has an anabolic role in the cell, tolerating a higher level of ATP than Zwf-1. Still, *zwf-2* is dispensable *in vivo* in

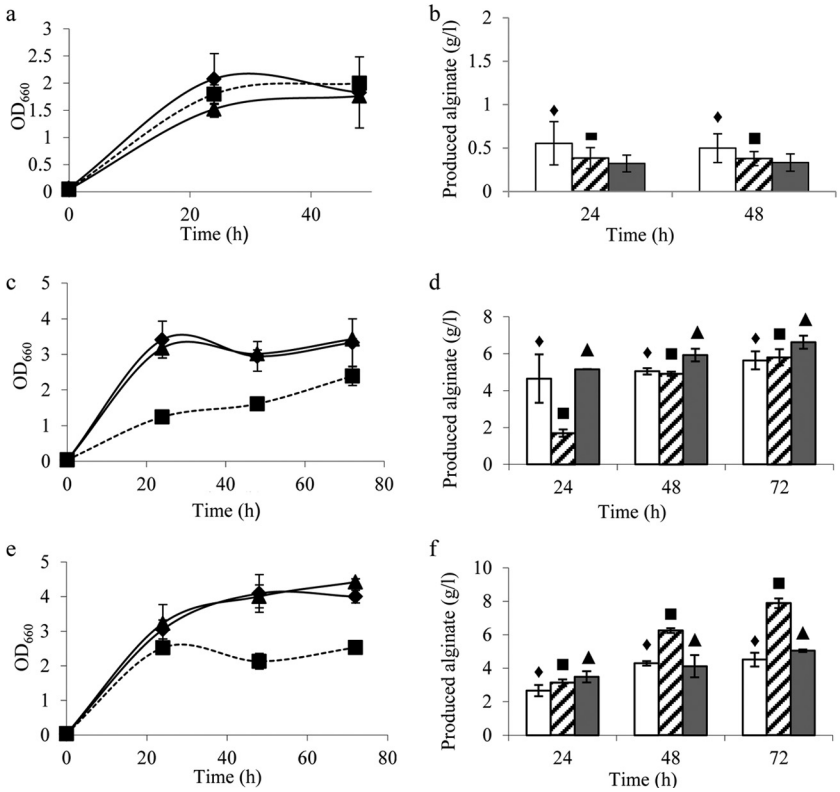

FIG 5 Effects of *zwf-1* disruption on growth and alginate production. *P. fluorescens* strains Pf201 (◆), Pf201 Δ*algC* Δ*zwf-1*::TnKB60 (■), and Pf201 Δ*algC*::TnKB60 MM27 (▲) were grown in minimal medium containing glucose (a and b), fructose (c and d), or glycerol (e and f). Growth and alginate data are shown as averages for at least two biological replicates. Error bars indicate standard deviations.

the presence of a functional *zwf-1* gene. Furthermore, the data show that for the wild-type strain, G6PDH activity is required for growth on fructose, and also for a normal growth rate when glycerol is used as the carbon source. Results similar to ours have been obtained with *Pseudomonas aeruginosa*, where a *zwf* mutant grew very poorly on fructose (16). *P. fluorescens* and *Pseudomonas putida* seem to have similar fructose metabolisms, and it has been shown that when *P. putida* grows on fructose, 52% of the sugar is channeled through the ED pathway, 34% through glycolysis, and 14% through the PP route (1). Thus, even if the flux through glycolysis is too low to allow for full growth, it is puzzling that hardly any growth was observed (Fig. 3b). The remaining part of the PP pathway should enable the synthesis of metabolites needed for growth (e.g., purines, pyrimidines, and histidine).

NADPH is one important metabolite resulting from G6PDH activity and is known to be important for surviving oxidative stress in *P. fluorescens* (17). Our *in vitro* data show that both Zwf-1 and Zwf-2 are able to reduce $NADP^+$. When the bacteria are cultivated on glucose, some NADPH is produced in the reaction catalyzed by 6-phosphogluconate dehydrogenase, while no NADPH is produced by the reactions required for the uptake and utilization of glycerol and fructose to produce glyceraldehyde-3-phosphate (GAP) (Fig. 1). This might explain some of the growth deficiencies of the *zwf-1 zwf-2* double mutant when it is cultivated on fructose, but not the differences observed between growth on fructose and growth on glycerol.

As shown in Fig. 1, FBP is at the branching point of the two pathways for fructose utilization. Based on the *P. putida* data (1), one could hypothesize that 66% of the FBP is converted to F6P, which might exceed the capacity of the PP pathway running in the reverse direction and possibly lead to an accumulation of F6P and G6P. Accumulation of sugar phosphates is known to hamper growth in *E. coli* (18), and this might be the case for *P. fluorescens* as well. In *E. coli*, synthesis of the glucose transporter is negatively regulated when such stress is experienced. However, so far, the only known regulation of the fructose transporter system in *Pseudomonas* is that gene expression is turned on in the presence of F1P (19). Alginate production can be assumed to drain away surplus F6P. Thus, if an increased concentration of hexose phosphates is contributing to the slower growth, the growth defects of strain Pf201 Δ*algC* Δ*zwf-1*::TnKB60 grown on fructose should be less severe than those for NCIMB 10525 Δ*zwf-1*, as was shown to be the case (compare Fig. 3b and 5c).

The periplasmic glucose dehydrogenase Gcd converts glucose to gluconate, resulting in acidification of the growth medium (20). Alginate production would be expected to decrease the pH further. Our results indicate that this affects both growth and alginate production negatively in the alginate-producing mutant Pf201 when grown on glucose, since the Δ*gcd* mutant reached higher final cell densities, avoided stress-induced cell death, and produced more alginate than strain Pf201. The increased alginate production observed for the *gcd* strain at 48 h may thus be caused partly by a higher number of living cells. However, this does not explain the difference in alginate production at 24 h, when the CFU counts of the two strains are similar (Fig. 4). No similar positive growth effect was observed for NCIMB 10525 Δ*gcd*,

which actually grew more slowly than the wild-type strain (Fig. 3a). In a non-alginate-producing strain, *gcd* inactivation could result in increased levels of G6P and F6P, which may, as described above, lead to the lower growth rate observed. Inactivation of *gcd* would necessitate that all glucose be converted by the glucokinase system, producing G6P. G6P may then be directly isomerized to F6P, which could partly explain the increased alginate production in the glucose-grown Pf201 Δ*gcd* mutant (Fig. 4b).

In a recent metabolome study of *P. fluorescens*, it was found that for cells grown on glycerol, an alginate-producing mutant had higher flux not only from F6P to alginate but also from F6P to G6P and through the ED pathway than the wild type (21). The latter data could explain why the *zwf-1* mutation was beneficial for alginate production in cells using glycerol as the carbon source (Fig. 5f), since this mutation would decrease the flow of carbon through the competing pathway.

Our results indicate that the rate of alginate biosynthesis depends on the amount of F6P available. Excessive amounts of hexose phosphates seem to be detrimental to the cell and appear to be alleviated by channeling the surplus hexose phosphates to alginate production. These results should be relevant for the production of many different polysaccharides and other secondary metabolites by bacterial cells. Strain Pf201 has been tested under conditions (in bioreactors) more relevant for commercial production processes and was found to be a high-level, stable producer of alginate (4). The additional effects of the *gcd* and *zwf-1* deletions reported here are therefore highly relevant for future development of processes for microbial alginate production.

ACKNOWLEDGMENTS

Susan Maleki and Mali Mærk were supported by grants from FUGE Midt-Norge and the Norwegian Research Council, respectively.

We thank Evy Renate Mellemsæther for constructing plasmid pEM1, Yi-Qian Sun for constructing plasmid pYQ1, and Karianne Bakkevig for constructing plasmid pKB64. We are grateful to Sergey B. Zotchev for helpful comments and discussions about this work.

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