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ORIGINAL PAPER

ATP limitation in a pyruvate formate lyase mutant of *Escherichia coli* MG1655 increases glycolytic flux to D-lactate

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Abstract A derivative strain of *Escherichia coli* MG1655 for D-lactate production was constructed by deleting the *pflB*, *adhE* and *frdA* genes; this strain was designated "CL3." Results show that the CL3 strain grew 44% slower than its parental strain under nonaerated (fermentative) conditions due to the inactivation of the main acetyl-CoA production pathway. In contrast to *E. coli* B and W3110 *pflB* derivatives, we found that the MG1655 *pflB* derivative is able to grow in mineral media with glucose as the sole carbon source under fermentative conditions. The glycolytic flux was 2.8-fold higher in CL3 when compared to the wild-type strain, and lactate yield on glucose was 95%. Although a low cell mass formed under fermentative conditions with this strain (1.2 g/L), the volumetric productivity of CL3 was 1.31 g/L h. In comparison with the parental strain, CL3 has a 22% lower ATP/ADP ratio. In contrast to wild-type *E. coli*, the ATP yield from glucose to lactate is 2 ATP/glucose, so CL3 has to improve its glycolytic flux in order to fulfill its ATP needs in order to grow. The *aceF* deletion in strains MG1655 and CL3 indicates that the pyruvate dehydrogenase (PDH) complex is functional under glucose-fermentative conditions. These results suggest that the pyruvate to acetyl-CoA flux in CL3 is dependent on PDH activity and that the decrease in the ATP/ADP ratio causes an increase in the flux of glucose to lactate.

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Introduction

D-Lactate demand has increased as consequence of its use in biopolymer applications. This compound can be transformed into a polymer that can be used as a substitute for oil-derived plastics and has the advantage of being biodegradable. The properties of polylactides can be controlled by using different proportions of each of the two lactate isomers [15]. Lactate can be produced in fermentative processes by bacteria using glucose as a raw material. Lactic acid bacteria have been used for this purpose; however, complex nitrogen sources are needed to obtain elevated productivities [14]. *Escherichia coli* is able to grow in mineral media under fermentative conditions, and it produces D-lactate as part of a mixture of products [2]. With the aim of increasing lactate production, metabolically engineered strains of *E. coli* have been generated and studied. The most commonly used strategy for strain generation involves inactivation of the pyruvate formate lyase (*pfl*) [5, 16, 21]. Under anaerobic conditions, 45% of the pyruvate flux is channeled through PFL [3]; acetyl-CoA and formate are produced by this enzyme. The *pfl* mutants can produce lactate as the main fermentation product, showing high sugar-to-lactate conversion yields, but their growth capacity is drastically hindered as a consequence of the limited flux to acetyl-CoA [16, 22]. These previous studies were done using derivatives of the *E. coli* strains W3110 and B. W3110 derivatives display poor growth in mineral media with glucose as the sole carbon source, so lactate productivities are low [16]. *E. coli* B derivatives engineered for D-lactate production rapidly ferment 10% (w/v) glucose but

complex media are required. A metabolic evolution strategy has been used to improve those strains, achieving high lactate yields and productivities of up to 2.88 g/L h in mineral media [7, 13, 17, 20, 21].

In this study we developed a D-lactate-producing strain derived from MG1655 that converts glucose to D-lactate with a 95% yield and the highest productivity reported so far in an unevolved strain. The ATP/ADP ratio, the pyruvate dehydrogenase contribution to acetyl-CoA formation, and the initial rate of glucose transport were studied in order to understand the high glycolytic flux present in MG1655 *ΔpflB ΔadhE ΔfrdA* (Fig. 1).

Materials and methods

Organisms and culture conditions

Escherichia coli MG1655 was used as the parental strain. The CL3 strain was constructed by deleting the *pflB*, *adhE* and *frdA* genes (Table 1). Deletion of *pflB* was done as previously reported [10]. Deletions of *adhE, frdA* and *aceF* were done with a chromosomal gene inactivation method using PCR products [4]. Primers were designed to amplify FRT-Kan-FRT from pKD4 or FRT-cat-FRT from pKD3 with 40–50 nucleotides homologous to the chromosome sequence in order to inactivate the target genes. All antibiotic markers were removed for the CL3 strain. Batch fermentations were performed employing a modified mineral AM1 medium [13] named AM2, supplemented with 40 g/L of glucose (pH adjusted to 7.0 with 2 N KOH), and (per liter) 2.63 g $(NH_4)_2HPO_4$, 0.87 g $NH_4H_2PO_4$, 1.0 mL $MgSO_4.7H_2O$ (1 M), 1.5 mL trace element solution, 1.0 mL KCl (2 M), 1.0 mL betaine HCl (1 M), and 100 mg citric acid. The trace element solution contained (per liter) 1.6 g $FeCl_3$, 0.2 g $CoCl_2{\cdot}6H_2O$, 0.1 g $CuCl_2$, 0.2 g $ZnCl_2{\cdot}4H_2O$, 0.2 g Na_2MoO_4, 0.05 g H_3BO_3 and 0.33 g $MnCl_2{\cdot}4H_2O$. Solid anaerobic cultures were carried out in anaerobic jars under a CO_2 atmosphere using a Gas Pak kit (Bectkon-Dickinson, Franklin Lakes, NJ, USA). M9 mineral medium [12] plates supplemented with glucose 2 g/L or a mixture of glucose 2 g/L and acetate 1 g/L were used; per liter: 6 g Na_2HPO_4, 3 g KH_2PO_4, 1 g NH_4Cl and 0.5 g NaCl. The following components were sterilized by filtration and then added (per liter of final medium): 2 mL of 1 M $MgSO_4{\cdot}7H_2O$, 1 mL of 0.1 M $CaCl_2$, 1 mL of 1 mg/mL thiamin HCl. Cultures were carried out in fleaker minifermentors [1] containing 200 mL of AM2 media, without aeration, at 37 °C, pH 7, 100 rpm and with 0.05 g/L of dry cellular weight as initial inoculum. pH was kept constant by automatically adding KOH 2 N. Rezasurin (1 μg/mL), a nontoxic redox indicator, became colorless in less than 1 h

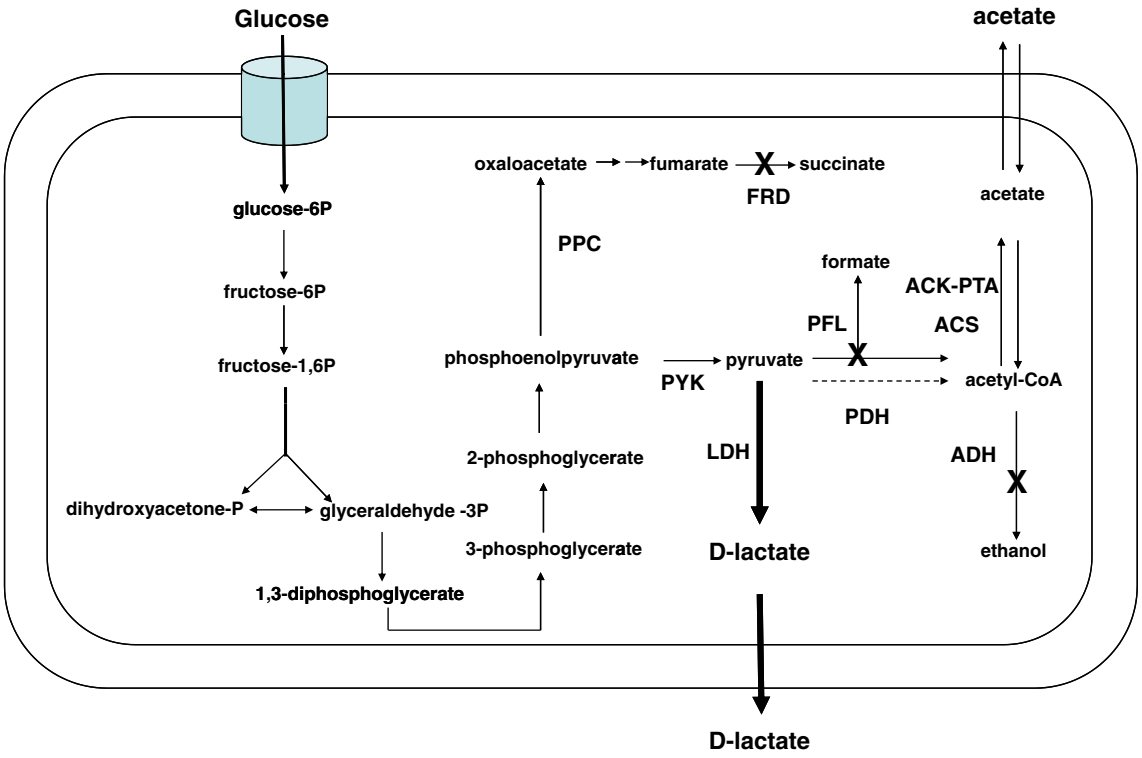

Fig. 1 Glucose to D-lactate metabolism in CL3. Deletions of competing pathways are marked with "*X*." *ACS*, acetyl-CoA synthetase; *ACK*, acetate kinase; *ADH*, alcohol dehydrogenase; *FRD*, fumarate reductase; *LDH*, lactate dehydrogenase; *PDH*, pyruvate dehydrogenase; *PFL*, pyruvate formate lyase; *PPC*, phosphoenolpyruvate carboxylase; *PTA*, phosphotransacetylase; *PYK*, pyruvate kinase

Table 1 Strains and plasmids used in this work

Strain	Relevant genotype	Source
MG1655	Wild type	Laboratory stock (this strain came from Dr. Blattner's laboratory, and shows no growth defects under anaerobic conditions)
CL3	MG1655 *ΔpflB ΔadhE ΔfrdA*	This work
CL3 aceF	CL3 *ΔaceF*::*cat*	This work
MG1655 aceF	MG1655 *ΔaceF*::*cat*	This work

when wild-type *E. coli* strains were cultivated under the abovementioned conditions. These conditions were maintained during the exponential and stationary phases of the fermentations. Although the cultures were not sparged with an inert gas in the headspace of the mini-fermentors, the behavior described above indicates that anaerobic conditions were maintained during most of the time covered by the batch cultures (from 24 to 30 h). All fermentations were carried out in triplicate (average and standard deviations are shown in plots and tables).

Analyses

Growth was determined spectrophotometrically as the optical density at 600 nm (DU-70, Beckman Instruments, Inc., Fullerton, CA, USA) and converted to dry cell weight per liter using a calibration curve (1 optical density = 0.37 g_{DCW}/L). Samples were centrifuged and the cell-free culture broth was frozen until analysis. Glucose, ethanol, acetic, formic, succinic, lactic, and pyruvic acids were determined by HPLC analysis (Waters U6 K, Millipore Co., Milford, MA, USA), using an Aminex HPX-87H ion exclusion column (300 × 7.8 mm; Bio-Rad Laboratories, Hercules, CA, USA), with a 5.0 mM H_2SO_4 solution used as the mobile phase (0.5 mL/min) at 60 °C, and with a photodiode array detector at 210 nm (Model 996, Waters, Millipore Co.) and a refractive index detector (Model 2410, Waters, Millipore Co.).

Measurements of the intracellular ATP and ADP concentrations

To measure the ATP/ADP ratio, a nucleotide extraction was performed using hot phenol, as reported by Koebmann et al. [9]. The ATP and ADP concentrations were then measured using the ENLITEN® ATP assay (PROMEGA, Madison, WI, USA), as recommended by the manufacturer. The concentration of ATP was measured first, and then the ADP content in the same sample was converted to ATP by adding 2 U of pyruvate kinase, 1 mM phosphoenolpyruvate (PEP), 5 mM KCl and 50 mM $MgCl_2$, and the concomitant increase in luminescence was recorded.

Measurement of the initial rates of glucose transport

Cells were harvested from fermentations in the mid log phase (5,000×*g* for 10 min at 4 °C), cooled on ice, washed in 1× AM2 salts, and dissolved in the same medium without sugars at an OD_{600} of 0.5. Since the cells were kept on ice before the transport experiments, metabolic activity was reduced, so it is considered that the amount of sugar transport protein is the same as that present during growth under fermentative conditions. For the [^{14}C]-glucose uptake assays, 540 µL of cell suspension were incubated for 15 min at 37 °C, and then the reaction was started by adding 60 µL of [^{14}C]-glucose (0.5 mM, 5 mCi/mmol). The reaction was kept at 37 °C with shaking, and 50 µL samples were taken at 0, 1, 2, 3, 5, and 10 min intervals. Samples were filtered immediately through membrane filters (pore size 0.22 µm) and washed three times with AM2 salts. Filters were dried and placed in vials with 5 mL of Ecolite scintillation cocktail (ICN Biomedicals, Costa Mesa, CA, USA). Radioactivity was measured in a scintillation counter (Beckman LS6000IC, Fullerton, CA, USA). Protein was measured with the Folin phenol reagent [11]. The [^{14}C]-glucose uptake rates were calculated from the initial linear data in a plot of intracellular [^{14}C]-glucose versus time. A Student's *t*-test with a *P* value of $\leq$0.05 was applied to each set of data in order to determine statistical differences in [ATP]/[ADP] ratios and glucose transport rates.

Results

Fermentation performance

Batch fermentations were carried out in AM2 mineral medium in controlled-pH mini-fermentors. Growth rate, biomass yield, glucose consumption rate, and fermentation products were measured. Figure 2 and Table 2 show that the CL3 strain grew at 0.24 h^{-1}, 44% slower than the parental strain MG1655 (0.44 h^{-1}). The parental strain produced up to 3 g/L of dry cell weight and consumed 43 g/L of glucose in 24 h. MG1655 showed

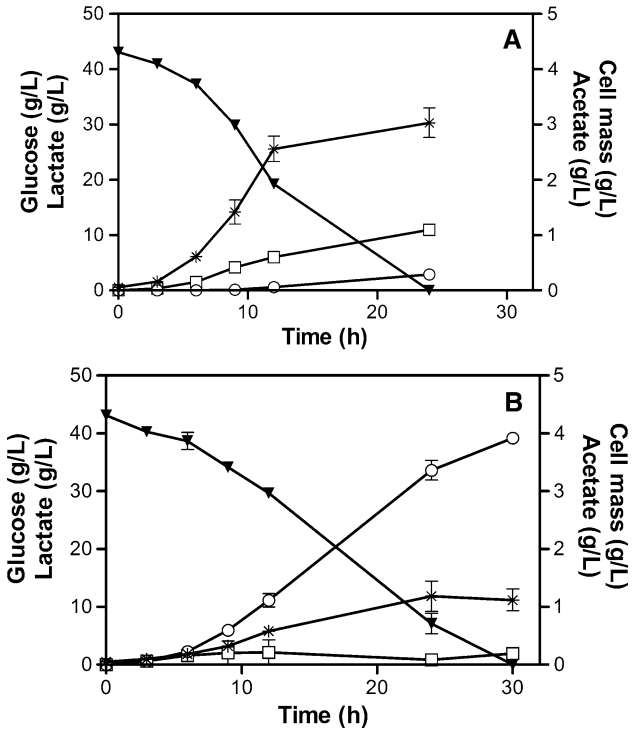

Fig. 2 Fermentation of 4% glucose by MG1655 (**A**) and CL3 (**B**). *Filled inverted triangles*, glucose; *asterisks*, cell mass; *circles*, lactate; *squares*, acetate

mixed acid fermentation, and the main products were acetate, formate, ethanol, lactate and succinate (Table 2). CL3 had a 61% lower final cell concentration than MG1655, but it consumed the 43 g/L of glucose in less than 30 h (Fig. 2). As a consequence of the fermentation pathway deletion, lactate yield was 95% (g/g) in CL3. No formate, succinate or ethanol were detected in the fermentation broth, and less than 1.0 g/L acetate was produced by CL3 at the exponential phase, which was partially consumed during the stationary phase. During the exponential growth phase, the specific glucose consumption rate was 2.8-fold higher in CL3 than in the parental strain (Table 2). In addition, the specific lactate production rate was 55-fold higher in CL3. The measured volumetric productivity of lactate for CL3 was 1.31 g/L h.

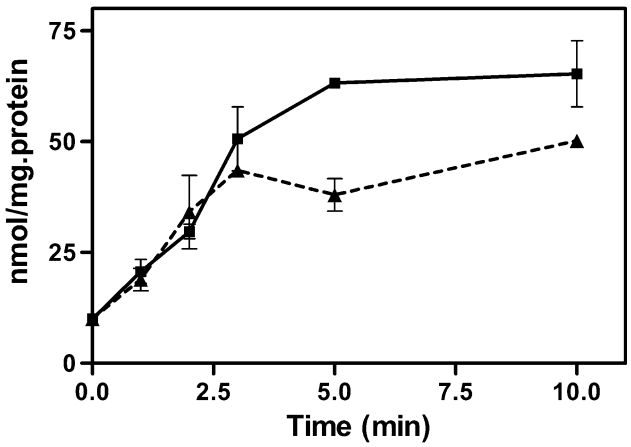

Fig. 3 Glucose transport rate kinetics for MG1655 (*straight line*) and CL3 (*dotted line*)

Measurement of glucose transport capacity

In order to investigate if the increase in the specific glucose consumption rate showed by CL3 had an effect on the rate of glucose transport, and considering that it is the first step of glycolysis, the glucose transport rate was measured (Fig. 3). A Student's *t*-test (*P* value of ≤ 0.05) showed that the glucose transport rates for CL3 (11.2 ± 0.46 $nmol_{Glc}/mg_{PROTEIN}$ min) and MG1655 (10.6 ± 0.21 $nmol_{Glc}/mg_{PROTEIN}$ min) were not significantly different. These results show that the glucose transport capacity of CL3 is not higher than the parental strain under the evaluated conditions, so glucose transport is not a limiting step in glucose consumption for the MG1655 strain.

ATP/ADP ratio

Homolactic fermentation from glucose yields only 2 ATP/mol of glucose, compared to mixed fermentation in wild-type *E. coli* that yields 3 ATP/glucose. The slower growth rate displayed by CL3 and the observed glycolytic flux increase may be attributed to a lower ATP pool [9, 22]. Table 2 shows that the ATP/ADP ratio in CL3 was 22% lower than the ratio in the wild-type strain. The Student's

Table 2 Specific growth rate (μ), specific glucose consumption rate (q_S), specific lactate production rate (q_P), fermentation products, lactate yield on glucose ($Y_{P/S}$), ATP/ADP ratio, and volumetric productivity (Q_{vol}) for MG1655 and CL3 strains

Strain	μ (h^{-1})	q_S (g/g h)	q_P (g/g h)	Lactate (g/L)[a]	Acetate (g/L)[a]	Formate (g/L)[a]	Ethanol (g/L)[a]	Succinate (g/L)[a]	$Y_{P/S}$ (g/g)	ATP/ADP (−)	Q_{vol} (g/L h)
MG1655	0.44 (0.018)	2.33 (0.20)	0.09 (0.05)	2.88	11.39	10.19	5.26	2.20	0.083	0.873 (0.143)	0.305
CL3	0.24 (0.004)	6.43 (0.36)	4.92 (0.63)	39.20	0.47	ND	ND	ND	0.95	0.685 (0.039)	1.31

Where appropriate, values in parentheses indicate the SD

ND, not detected

[a] Maximum amount of product detected

t-test shows that this difference is statistically significant (*P* value of ≤0.05).

PDH contribution to acetyl-CoA formation in CL3

In order to quantify the contribution of pyruvate dehydrogenase (PDH) complex activity to acetyl-CoA formation from pyruvate in CL3, the gene *aceF* was deleted in both the MG1655 and the CL3 strains (Table 1, Fig. 1). Phenotypic analyses were carried out in anaerobic jars using solid mineral media with glucose or glucose and acetate mixtures (see "Culture conditions" for details). Results showed that *aceF* deletion caused acetate auxotrophy under aerobic conditions for both strains (MG1655 and CL3). Under anaerobic conditions, MG1655 *ΔaceF* was able to grow without acetate supplementation, but CL3 *ΔaceF* was not able to grow in glucose as the sole carbon source. These results indicate that PDH contributes to acetyl-CoA formation in CL3 under fermentative conditions.

Discussion

In contrast to other D-lactate production strains reported elsewhere, CL3 shows several advantages: (a) a growth rate of 0.24 h^{-1} and a maximal cell concentration of 1.2 g/L; (b) a 95% lactate yield from glucose; (c) less than 1 g/L of acetate as byproduct; and (d) a high glycolytic flux. Under the evaluated conditions, CL3 achieved a volumetric productivity of 1.31 g/L, which is the highest reported for an unevolved strain of *E. coli* in batch fermentation. The effects of *pfl* knockout on enzyme activities, intracellular metabolite concentrations and production yields under microaerobic conditions have been previously studied using *E. coli* BW25113 derivatives [22]. Results show that the ATP/AMP ratio in the *pfl* knockout is 44% lower compared to the progenitor strain, and glycolytic flux and the activity levels of glycolytic pathway enzymes are increased. It has been proposed that the ACK-PTA pathway may be active in the direction that produces acetyl-CoA from acetate while consuming 1 mol of ATP (see Fig. 1) [22]. The acetyl-CoA pool under microaerobic conditions was the same as in the parental strain, but cell yield was lowered. Low succinate formation was also found, even upon supplying CO_2, which indicates that in the phosphoenol pyruvate node the pyruvate kinase reaction showed a higher flux than phosphoenol pyruvate carboxylase; hence the ATP demand is favored over other reactions, favoring homolactic fermentation [22].

In contrast to aerobic conditions, where pyruvate dehydrogenase (PDH) is more active, in our experiments the only pyruvate dissimilation pathway is D-lactate fermentation. It has been demonstrated that a low ATP/ADP ratio increases glycolytic flux under aerobic conditions [9]. The lower ATP/ADP ratio found in CL3 when compared to the parental strain, and the conversion of acetate to acetyl-CoA by the ACK-PTA pathway, as has previously been proposed for *pfl* mutants, may be the causes of the 2.8-fold increase in the specific glucose consumption rate shown by CL3. The slow growth rates and reduced cell yields of previously reported *pfl* mutants of *E. coli* have been attributed to the lack of flux of pyruvate to acetyl-CoA and to the lower ATP yield in homolactic strains [16, 22]. Using *E. coli* B, it has previously been shown that there is ∼5% residual PDH activity under anaerobic conditions relative to aerobic culture conditions [18]. In addition, it has been shown that PDH can carry out the pyruvate to acetyl-CoA conversion in *pfl* mutants that have evolved to produce ethanol with the native *E. coli* pathway [8, 18]. These previous results show the capacity of PDH to carry out pyruvate to acetyl-CoA conversion, even under nonaerobic conditions. Our results show that the residual activity of PDH under anaerobic conditions, possibly due to incomplete transcription repression, may be responsible for acetyl-CoA formation in CL3; this characteristic may constitute the main phenotypic difference between MG1655 derivatives and other parental strains used in the development of lactic-acid-producing *E. coli* strains.

The transport rate measurements experiments revealed that the low ATP/ADP ratio has no influence on the *E. coli* glucose transport capacity; hence, the higher glucose consumption rate shown by CL3 may be dependent on higher activities of glycolytic enzymes [22], and may perhaps be due to changes in feedback regulation, as PFK and PYK enzymes are susceptible to ATP, phosphoenol pyruvate or pyruvate inhibition [6].

The production of lactate by *pfl* mutants of *E. coli* has been widely reported [13, 16, 19–22]. W3110-based biocatalysts with *pfl* and other fermentative gene deletions (*frd* and *adhE*)—strains SZ40 and SZ58—were able to produce optically pure D-lactate in mineral media with a 98% yield from glucose. However, as previously mentioned for *pfl* mutants, cell yield is drastically reduced (0.495 g/L), and so the volumetric productivity is also reduced (0.66 g/L h) [16]. Adaptive evolution has been used as a method to overcome the growth limitations resulting from *pfl* deletion. D-Lactate-producing derivatives of *E. coli* B have been developed using these approaches. Strain SZ132 was obtained using LB medium with 10% glucose. No results for the initial strain in mineral media are reported [21]. Further improvements were made by using several rounds of adaptive evolution, adding 1 mM of betaine [17] and deleting genes from the methylglyoxal bypass pathway. High lactate yields and productivities, up to 2.88 g/L h in mineral media, were reported [7, 17] for the evolved strain. Our work shows that, compared with other progenitor strains,

MG1655 has a better genetic background for D-lactate production. The glycolytic and lactate fluxes achieved by CL3 are the highest reported for an unevolved *E. coli pfl* mutant. These characteristics make CL3 a good candidate for use in D-lactate production.

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