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Short Communication

Construction and use of new cloning vectors for the rapid isolation of recombinant proteins from *Escherichia coli*

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Abstract

We describe the construction and use of two sets of vectors for the over-expression and purification of protein from *Escherichia coli*. The set of pTEV plasmids (pTEV3, 4, 5) directs the synthesis of a recombinant protein with a N-terminal hexahistidine (His_6) tag that is removable by the tobacco etch virus (TEV) protease. The set of pKLD plasmids (pKLD66, 116) directs the synthesis of a recombinant protein that contains a N-terminal His_6 and maltose-binding protein tag in tandem, which can also be removed with TEV protease. The usefulness of these plasmids is illustrated by the rapid, high-yield purification of the 2-methylcitrate dehydratase (PrpD) protein of *Salmonella enterica*, and the 2-methylaconitate isomerase (PrpF) protein of *Shewanella oneidensis*, two enzymes involved in the catabolism of propionate to pyruvate via the 2-methylcitric acid cycle.

Keywords: TEV protease-cleavable tags; Rapid protein purification; Propionate catabolism; 2-Methylcitric acid cycle enzymes; 2-Methylaconitate isomerase; 2-Methylcitrate dehydratase

1. Introduction

The synthesis of the first biologically functional bacterial plasmid (Cohen et al., 1973) signaled the beginning of molecular cloning and recombinant DNA technology. With the advent of recombinant DNA technology, researchers were first able to selectively increase the expression of single genes, which in turn increased yields of protein purifications. However, purification of proteins in their native form is still a difficult and time-consuming process.

Not until the advent of affinity tag purification did protein purification become a faster and more efficient process. Development of affinity tags such as the maltose-binding tag (di Guan et al., 1988; Maina et al., 1988), the polyhistidine tags of the pET vector series, (Rosenberg et al., 1987; Studier and Moffatt, 1986; Studier et al., 1990), and the IMPACT® intein chitin-binding tag (Chong et al., 1997,1998) allowed for more efficient and rapid

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purification of diverse proteins at yields much higher than would have been possible through native purification. The development of instruments such as the Maxwell™ 16 Instrument and the Maxwell™ 16 Polyhistidine Protein Purification Kit (Promega), have taken protein purification a step further by now allowing for the automated purification of up to 16 polyhistidine tagged proteins in less than one hour.

While the addition of affinity tags allow for ease of purification, it does render the protein into a non-native state and the affinity tag can often hamper subsequent work with the protein. While the IMPACT® system requires cleavage of the protein from the tag as an elution step, other systems require a subsequent cleavage and purification to remove the affinity tag. One method of tag removal is through the use of the tobacco etch virus (TEV) protease. TEV protease has become one of the proteases of choice for cleaving fusion proteins due to its high degree of specificity, its resistance to many protease inhibitors used in protein purification, and the ease of separation of both the protease and affinity tag from the protein of interest (Parks et al., 1994). Additionally, improvement to the ability to purify large amounts of TEV protease (Blommel and Fox, 2007; Lucast et al., 2001; van den Berg et al., 2006) can make the TEV protease a relatively inexpensive option for cleavage of fusion proteins when compared to other commercially available options.

Previous work has established that various protein fusion vectors featuring rTEV protease cleavage sites are viable for the purification of high levels of recombinant protein through high-throughput approaches (Dummler et al., 2005; Korf et al., 2005) or via ligation independent cloning (Cabrita et al., 2006). Here, we report on the construction of two new series of vectors to the growing family of rTEV-cleavable protein fusion vectors that allow for efficient purification of recombinant bacterial proteins. The pTEV series is based off the Novagen pET vector series and incorporates a TEV protease cleavage site as well as an improved variety of restriction endonucleases sites to increase cloning options. The pKLD series utilizes the pET vector backbone while incorporating both a polyhistidine tag as well as the maltose-binding tag from the New England Biolabs pMAL vector series. The pKLD series also have a TEV protease cleavage site and improved multiple cloning sites.

2. Results and discussion

2.1. pTEV plasmids direct the synthesis of proteins fused to a hexahistidine (H_6) N-terminal tag

pTEV vectors used to overproduce proteins with TEV-cleavable N-terminal His_6 tags were constructed by modifying the Novagen ketosteroid isomerase (KSI) fusion plasmid pET-31b(+). The KSI coding sequence was removed by digestion restriction enzymes NdeI and Bpu1102I(EspI). A hexahistidine (His_6) tag coding sequence, a seven amino acid spacer sequence, and a SpeI site were introduced at the NdeI and Bpu1102I sites with the rTEVLink1 DNA fragment (Table 1, supplemental material). This temporary plasmid was designated pKLD35. A rTEV protease cleavage site was inserted into plasmid pKLD35 at the SpeI and Bpu1102I sites with the rTEVLink2 DNA fragment (Table 1, supplemental material). The resulting plasmid was named pTEV4[1] (Fig. 1). If the NheI site is used for cloning the coding sequence of the gene of choice, after TEV protease cleavage the resulting protein only has three additional amino acids (Gly-Ala-Ser) attached to the N-terminus (Fig. 1).

To increase the usefulness of plasmid pTEV4, the number of restriction sites available in the multiple cloning site (MCS) was increased. The TEV MCS DNA fragment (Table 1) was ligated into plasmid pTEV4 at the NheI and Bpu1102I(EspI) restriction sites. The resulting plasmid was named pTEV3 (Fig. 1).

2.2. Moving genes from pET vectors into pTEV plasmids

The NdeI site of pTEV3 was inactivated thorough digestion and end filling. A new NdeI site was introduced with the TEVStartMCS DNA fragment (Table 1, supplemental material) at the NheI and EcoRI restriction sites. This final plasmid was given the name pTEV5 (Fig. 1). Use of the NdeI site as a 5′ cloning site results in four additional residues (Gly-Ala-Ser-His) at the N-terminus of the protein after TEV protease cleavage (Fig. 1). While the use of this site adds several additional residues, the NdeI site is a common 5′ cloning site

[1] *Accession #:* pTEV4, EU337979; pTEV3, EU337980; pTEV5, EU337981; pKLD66, EU337982; pKLD116, EU337983.

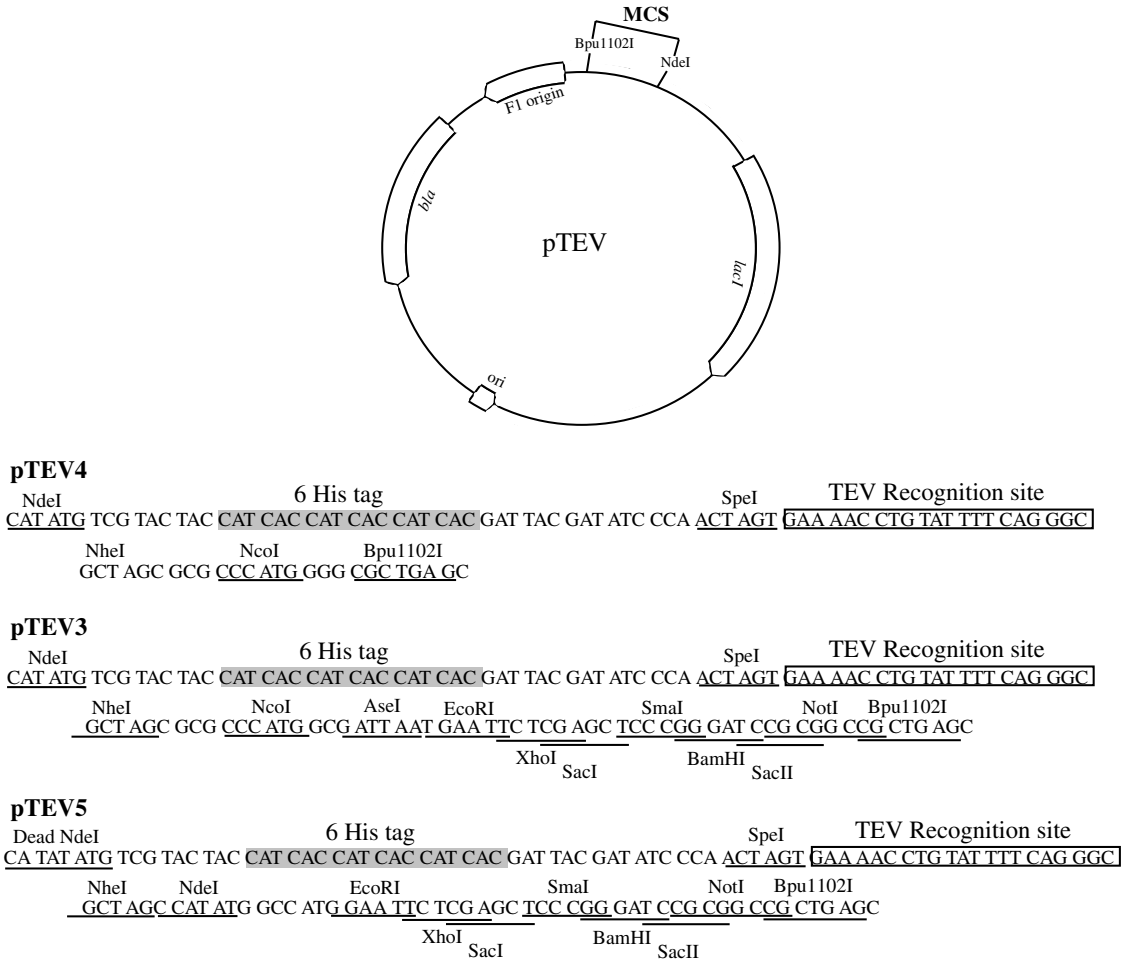

Fig. 1. Map of pTEV plasmids. Plasmid schematic is shown with relevant genetic elements. Plasmid sequence between the NdeI and Bpu1102I restrictions sites is shown below for each individual plasmid.

in other pET vectors and addition of it to the pTEV vectors allows for the quick removal of an insert from pET vectors and insertion into the pTEV vector series.

2.3. Use of His_6-TEV vectors to isolate the 2-methylaconitate isomerase (PrpF) enzyme of Shewanella oneidensis

To assess the usefulness of pTEV plasmids, we expressed the *prpF* gene from *Shewanella oneidensis* after cloning it into the NheI and NcoI sites of plasmid pTEV4. The resulting plasmid (pPRP196) was transformed into *E. coli* strain Bl21(λDE3) (New England Biolabs), a strain widely used to overproduce recombinant proteins.

His_6-PrpF was purified from clarified cell-free extracts using the Novagen His-Bind® nickel affinity chromatography kit following manufacturer's instructions and the results can be seen in Fig. 2. Enzymatic assays were performed to determine that the protein was active and the average yield of protein from purification, after rTEV cleavage, was 15 mg/g of wet cells. The purity of the protein was not determined as no contaminating bands could be observed in SDS–PAGE gels, so the protein was assumed to be greater than 95% pure.

Along with pTEV4, both pTEV3 and pTEV5 have been used to purify large amounts of highly purified protein with an average protein yield after removal of the tag, varying from 10 to 20 mg protein per gram of wet cells, depending upon the

Table 1
Oligonucleotides used to construct pTEV plasmids. The SpeI sites in rTEVLink1F and rTEVLink1R are in bold type. The sequence introduced into pMAL-c2x with the pMALHisMut mutagenic oligonucleotides is underlined; all other sequence is complementary

Name	Sequence 5′ → 3′
rTEVLink1F	TATGTCGTACTACCATCACCATCACCATCACGATTACGATATCCCAACTAGTGGCGC
rTEVLink1R	TCAGCGCCACTAGTTGGGATATCGTAATCGTGATGGTGATGGTGATGGTAGTACGACA
rTEVLink2F	CTAGTGAAAACCTGTATTTTCAGGGCGCTAGCGCGCCCATGGGGCGC
rTEVLink2	TCAGCGCCCCATGGGCGCGCTAGCGCCCTGAAAATACAGGTTTTCA
TEV MCS 1	CATGGCGATTAATGAATTCTCGAGCTCCCGGGATCCGCGGCCGC
TEV MCS 2	TGAGCGGCCGCGGATCCCGGGAGCTCGAGAATTCATTAATCGC
TEV Start MCS 1	CTAGCCATATGGCCATGG
TEV Start MCS 2	AATTCCATGGCCATATGG
pMALHisMutF	CCAACAAGGACCATAGCATATGGGCAGCCATCACCATCACCATCACTCCGGTAAAA TCGAAGAAGGTAAACTGG
pMALHisMutR	CCAGTTTACCTTCTTCGATTTT ACCGGAGTGATGGTGATGGTGATGGCTGCC CATAT GCTATGGTCCTTGTTGG
MCSInsertF	CGAGCGGAACCGCCTCGGGCGGTGCAACCACGTCAGAGAATCTCTACTTCCAAGGTACCTCGGACT
MCSInsertR	CTAGAGTCCGAGGTACCTTGGAAGTAGAGATTCTCTGACGTGGTTGCACCGCCCGAGGCGG TTCCGCTCGAGCT
NEW66f	CGAGCGGAACCGCCTCGGGCGGTGCAACCACTAGTGAGAATCTCTACTTCCAAGGCCTT AGCAGGTGCATGTGGA
	CGTCCCGGGCTAGCCATGGCCTGCA
NEW66r	GGCCATGGCTAGCCCGGGACGTCCACATGCACCTGCTAAGGCCTTGGAAGTAGAGATTCTCACTAG TGGTTGCACC
	GCCCGAGGCGGTTCCGCTCGAGCT

individual protein purified. These proteins have been used in both enzymatic and crystallographic studies which helps demonstrate the functionality of these plasmids (Garvey et al., 2007; Gray and Escalante-Semerena, 2007; Lewis and Escalante-Semerena, 2007; St Maurice et al., 2007).

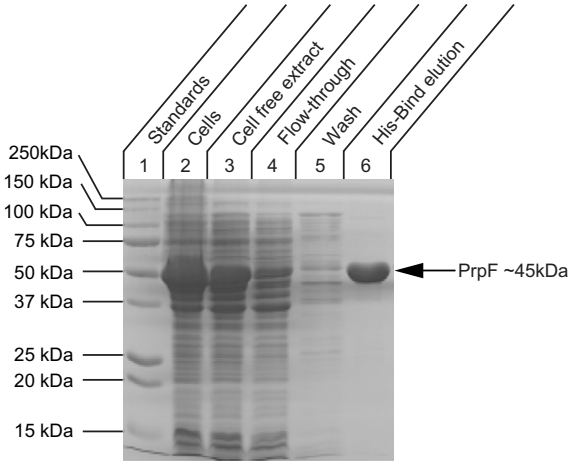

Fig. 2. Purification of rTEV-cleavable His-tagged 2-methylaconitate isomerase (PrpF) enzyme. A culture of the over expression strain was grown at 37 °C in LB supplemented with ampicillin to an OD_{650} of ~0.6 and induced with IPTG (0.3 mM) for approximately 18 h. Cells were harvested and protein was purified using Novagen's His-Bind® Kit and manufacturer's procedures. A 12% SDS–PAGE gel was loaded with 20 μg of lysed cells (lane 2), soluble cell-free extract (lane 3), column flow-through (lane 4), and 10 μg of wash fraction (lane 5) and eluted protein (lane 6). Bio-Rad's Precision Plus Protein™ Standard were loaded in lane 1. The gel was stained with Coomassie brilliant blue.

2.4. Construction of His-MBP-TEV vectors

In addition to the modified pET vectors, we engineered a maltose-binding protein (MBP) fusion vector to include a rTEV protease cleavage site. First, a His_6 tag was introduced 5′ from the *malE* gene in vector pMAL-c2x (NEB). The pMALHisMut DNA fragment (Table 1, supplemental material) was ligated to the plasmid 5′ to the MalE gene. This temporary plasmid was given the name pKLD54. To introduce a rTEV cleavage site and amino acid spacer downstream from the *malE* gene, plasmid pKLD54 was cut with restriction endonucleases SacI and XbaI and the DNA fragment MCSInsert (Table 1, supplemental material) was ligated into the plasmid. The resulting plasmid was named pKLD55. pKLD55 was cut with restriction endonucleases NdeI and HindIII to remove the His_6-*malE*-TEV fragment, which was inserted into pET21a(+) at the same sites. The resulting plasmid was named pKLD66 (Fig. 3). To increase the number of restriction sites in the multiple cloning site, the NEW66 DNA fragment

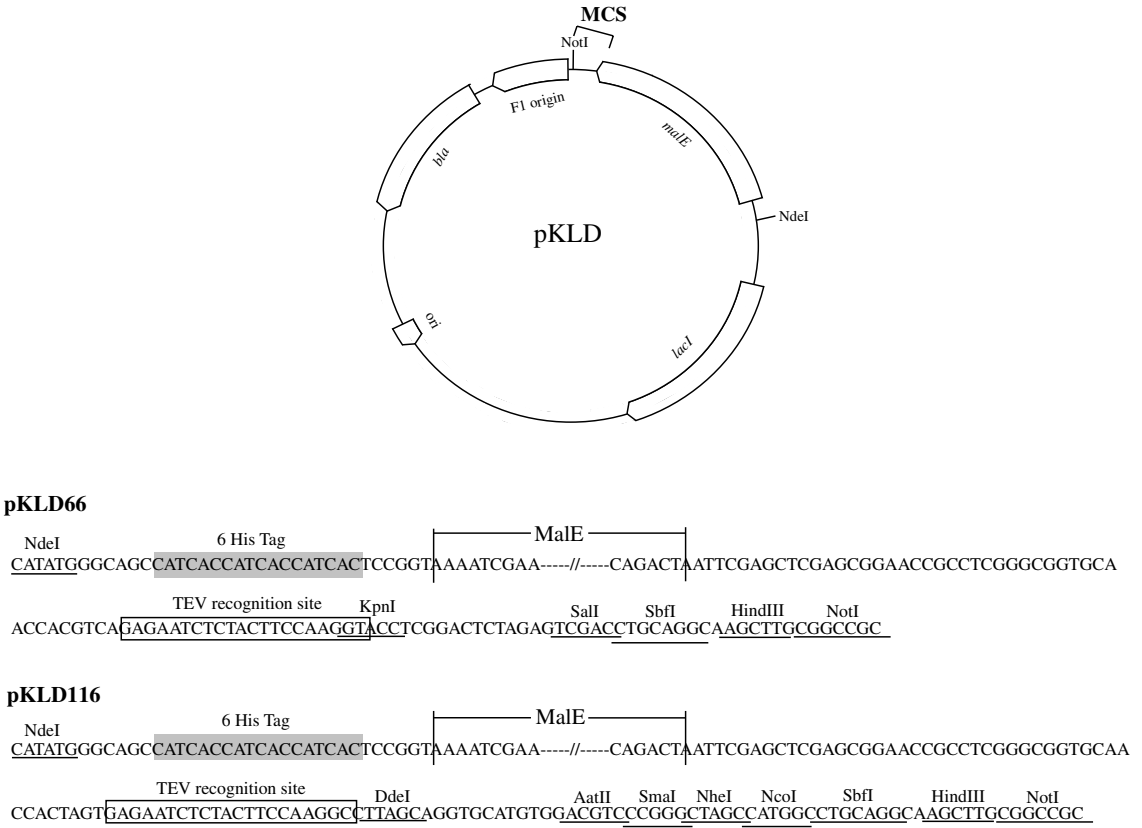

Fig. 3. Map of pKLD plasmids. Plasmid schematic is shown with relevant genetic elements. Plasmid sequence between NdeI and NotI restriction sites is shown for each individual plasmid. The *male* gene is shown in a truncated form for space considerations.

(Table 1, supplemental material) was ligated into plasmid pKLD66 at the SacI and SbfI sites. The resulting plasmid was named pKLD116 (Fig. 3). In plasmid pKLD66, use of the KpnI restriction site as a 5′ cloning site results in recombinant protein with two additional residues (Gly-Thr) at the N-terminus of the protein (Fig. 3). The StuI site in plasmid pKLD116 permits blunt-end cloning at the 5′ end and can, depending on the restriction enzyme used to cut the insert, result in a single glycine added to the N-terminus of the protein.

2.5. Isolation of 2-methylcitrate dehydratase (PrpD) protein of Salmonella enterica using His-MBP-TEV vectors

To assess the usefulness of the MBP tag in the isolation of recombinant proteins, the 2-methylcitrate dehydratase gene (*prpD*) gene of *S. enterica* was cloned into the KpnI and NotI sites of plasmid pKLD66 to yield plasmid pPRP222. The latter was introduced by transformation into *E. coli* strain Bl21(λDE3), the *prpD* gene was expressed, and the fusion protein was purified (Fig. 4).

The yield of purified protein was high (~25 mg of recombinant protein per gram of wet cells) and could be increased based on the large amount of recombinant protein that was visible in the column flow-through (Fig. 4, lane 4). Other experiments have shown that this protein was soluble and active, suggesting that in all probability the binding capacity of the column used in this experiment was exceeded. Recombinant protein was digested with rTEV protease, purified as described by Blommel and Fox (2007), and the resulting PrpD protein was purified from the His_6-MBP tag and His_6-rTEV (Fig. 5). Approximately 90% of PrpD protein was recovered from the rTEV digestion reaction, and assays indicated that the enzyme was active. The high-yield of recovery of cleaved PrpD protein indicated that the cleavage

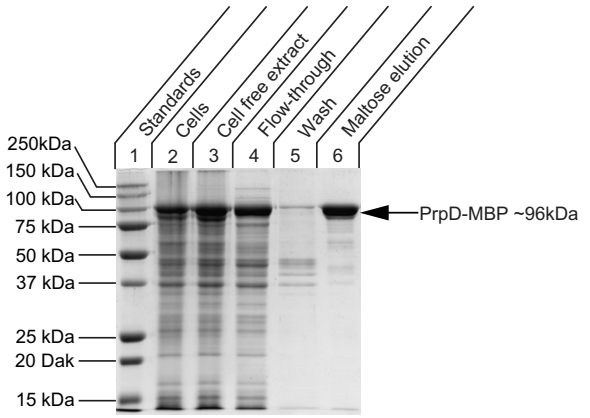

Fig. 4. Purification of MBP-tagged 2-mehtylcitrate dehydratase (PrpD) protein. Cells were grown in LB supplemented with ampicillin at 37 °C to an OD_{650} of ~0.7 and induced with IPTG for 16 h. Cells were harvested by centrifugation and broken with Novagen's BugBuster® plus lysozyme (1 mg/ml). Protein was purified using Bio-Rad Laboratories Amylose Resin High Flow as per manufacturer's protocols. A 12% SDS–PAGE gel was loaded with 20 μg of cell slurry (lane 2), cell-free extract (lane 3), and column flow-through (lane 4), column wash fraction (lane 5) and 3 μg of eluted protein (lane 6). Bio Rad's Precision Plus Protein™ Standards were loaded in lane 1. The gel was stained with Coomassie brilliant blue.

of the MBP tag and recovery was a very efficient process.

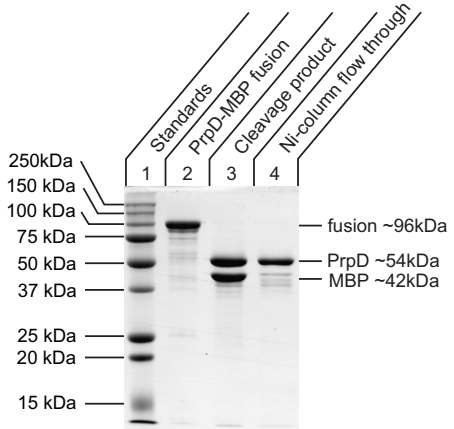

Fig. 5. Cleavage and purification of recombinant His_6-MBP-PrpD protein. Lane 1: Bio-Rad Precision Plus Protein™ Standards; lane 2: recombinant His_6-MBP-PrpD protein; lane 3: PrpD protein after rTEV cleavage; lane 4: purified PrpD protein. Cleavage of recombinant His_6-MBP-PrpD with rTEV protease was performed at a 50:1 mg:mg ratio during overnight dialysis at 4 °C against Tris–HCl buffer (20 mM, pH 7.9 at 25 °C) containing NaCl (200 mM), DTT (5 mM). DTT was removed by dialysis Tris–HCl buffer (20 mM, pH 7.9 at 25 °C) containing 400 mM NaCl and the cleaved protein was purified by running over a Novagen His-Bind® Resin column to remove both the His_6-MBP tag and His_6-rTEV protease.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.plasmid.2008.01.001.

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