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ORIGINAL PAPER

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A wide-range integrative yeast expression vector system based on *Arxula adeninivorans*-derived elements

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Abstract An *Arxula adeninivorans*integration vector was applied to a range of alternative yeast species including *Saccharomyces cerevisiae*, *Debaryomyces hansenii*, *Debaryomyces polymorphus*, *Hansenula polymorpha* and *Pichia pastoris*. The vector harbours a conserved *A. adeninivorans*-derived 25S rDNA sequence for targeting, the *A. adeninivorans*-derived *TEF1*promoter for expression control of the reporter sequence, and the *Escherichia coli*-derived *hph* gene conferring resistance against hygromycin B for selection of recombinants. Heterologous gene expression was assessed using a green fluorescent protein (GFP) reporter gene. The plasmid was found to be integrated into the genome of the various hosts tested; recombinant strains of all species exhibited heterologous gene expressions of a similar high level.

Keywords Green fluorescent protein · General yeast vector · Heterologous gene expression · 25S rDNA · Yeast

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Introduction

Yeasts are excellent hosts for the production of recombinant proteins, offering ease of genetic manipulation and cultivation to high cell density at a fast growth rate [8, 9, 15, 28, 34]. As eukaryotes, they are able to perform complex posttranslational modifications, thus producing foreign proteins that are often identical or very similar to native products of mammalian sources [8, 9, 11, 33, 37, 39]. The first yeast species to be employed for the production of foreign proteins was *Saccharomyces cerevisiae*. So far, a wide range of heterologous proteins has been produced in this host [15, 34, 36]. However, the disadvantages of the *S. cerevisiae* system quickly became apparent, limiting its general use in biotechnology: glycoproteins are often over-glycosylated and terminal mannose residues in N-linked glycans are added by an α1,3 bond that is suspected to be allergenic [13, 16]. In many instances, relatively low yields of recombinant products are encountered. The narrow substrate specificity prevents diverse fermentation design [3, 28]. Therefore, biotechnological application has been extended to alternative yeast species that have been developed as expression systems. A growing list of such non-conventional yeasts successfully employed in heterologous protein synthesis now includes, among others, *Hansenula polymorpha* [7—9, 26], *Pichia pastoris* [4], *Candida boidinii* [8], *Schwanniomyces occidentalis* [27], *Pichia stipitis* [27], *Yarrowia lipolytica* [17, 26], *Kluyveromyces lactis* [1, 26], and *Arxula adeninivorans* [32].

Since an optimal system of general use obviously does not exist, a suitable host has to be defined for each heterologous gene to be expressed. It would be useful to assess several selected organisms in parallel for criteria such as appropriate protein processing or secretion in a particular strain development. The availability of a vector that could be employed to transform the various candidates in parallel would greatly facilitate such

comparisons. As a prerequisite, such a vector has to contain a targeting element suitable for all test species and a promoter that drives heterologous gene expression in all these organisms. In the following study, we describe such a vector. It contains an *A. adeninivorans* 25S rDNA fragment for targeting, and the *A. adeninivorans*-derived *TEF1* promoter for control of heterologous gene expression.

In the present study, this vector was tested in a range of yeast species including *A. adeninivorans*, *S. cerevisiae*, *Debaryomyces hansenii*, *Debaryomyces polymorphus*, *H. polymorpha* and *P. pastoris*.

Materials and methods

Strains and media

Escherichia coli TOP 10 [F′, *mcrA*, Δ(*mrr-hsdRMS-mcrBC*), Φ *80ΔlacZ-ΔDM15*, *nupG*, Δ*lacX74*, *deoR*, *recA1*, *araD139*, Δ(*ara*, *leu*), *7697*, *galU*, *galK*, λ^-, *rpsL*, *endA1*] (Invitrogen, Carlsbad, Calif.), served as host strain for bacterial transformation and plasmid isolation. The strain was grown in LB medium supplemented with ampicillin (50 μg ml^{-1}; AppliChem, Darmstadt, Germany) when required for selection.

The yeast strains *A. adeninivorans* LS3 [21], *A. adeninivorans* 135 [38], *D. hansenii* H158 (provided by the strain collection of UFZ, Leipzig, Germany), *D. polymorphus* H120 (provided by the strain collection of UFZ, Leipzig, Germany), *P. pastoris* GS115 (*his4*; Invitrogen) and the *H. polymorpha* RB11 (*odc1*; [10]) as well as *S. cerevisiae* C13ABYS86 (MATα *leu2 ura3 his pra1 prb1 prc1 cps*; [14]) were used as hosts. All strains were grown either under non-selective conditions in complex medium (YEPD) or under selective conditions in a yeast minimal medium (YMM) supplemented with 2% glucose [29, 35]. Cultivation was performed at 30°C.

Agar plates were prepared by adding 1.6% (w/v) agar to medium. Hygromycin B (Roche Diagnostics, Mannheim, Germany) was added as 150–400 μg ml^{-1}, when required for selection.

Isolation and characterisation of nucleic acids

Chromosomal DNA of *A. adeninivorans* LS3, *A. adeninivorans* 135, *D. hansenii*H158, *D. polymorphus* H120, *H. polymorpha* RB11, *P. pastoris* GS115 and *S. cerevisiae* C13ABYS86 was isolated by the procedure of Kunze et al. [22]. DNA fragments for probing were isolated from an agarose gel applying the QIAEX II kit (Qiagen, Valencia, Calif.). The isolated fragments were labelled with [α-^{32}P]dATP (Amersham, Little Chalfont, UK) using a random primer system (Gibco, Rockville, Md.) and employed as DNA hybridisation probes.

For Southern transfer 1 μg DNA was separated on a 0.8% agarose gel and transferred to nitrocellulose (Hybond N, Amersham) by vacuum blotting. Hybridisation to a ^{32}P-labelled probe was carried out at 65°C and 5× SSC. Blots were exposed to Hyperfilm-MP (Amersham) at −80°C.

Re-transformation of *E. coli* with total yeast DNA followed the procedure described by Kunze et al. [22].

Yeast transformation

A. adeninivorans LS3, *A. adeninivorans* 135, *D. hansenii* H158, *D. polymorphus* H120, *H. polymorpha*RB11, *P. pastoris* GS115 and *S. cerevisiae* C13ABYS86 were transformed according to Rösel and Kunze [32] and Dohmen et al. [6]. Stable transformants were obtained after a sequence of passages on selective and non-selective media. After transformation, hygromycin-B-resistant colonies were selected on YEPD agar plates supplemented with 150–400 mg l^{-1} hygromycin B (200 mg l^{-1} for *A. adeninivorans* LS3 and 135, 250 mg l^{-1} for *D. hansenii* H158 and *D. polymorphus* H120, 400 mg l^{-1} for *H. polymorpha* RB11, 150 mg l^{-1} for *P. pastoris* GS115 and *S. cerevisiae* C13ABYS86). Single colonies were isolated and grown on YEPD medium + hygromycin B at 30°C for 2 days. This step was repeated three times before the cells were plated on non-selective YEPD agar and grown for 3–5 days at 30°C. A single colony from each transformant was isolated and defined as a strain.

Plasmid construction

The integrative vector pAL-HPH-TEF-GFP (Fig. 1) was used for the expression of the green fluorescent

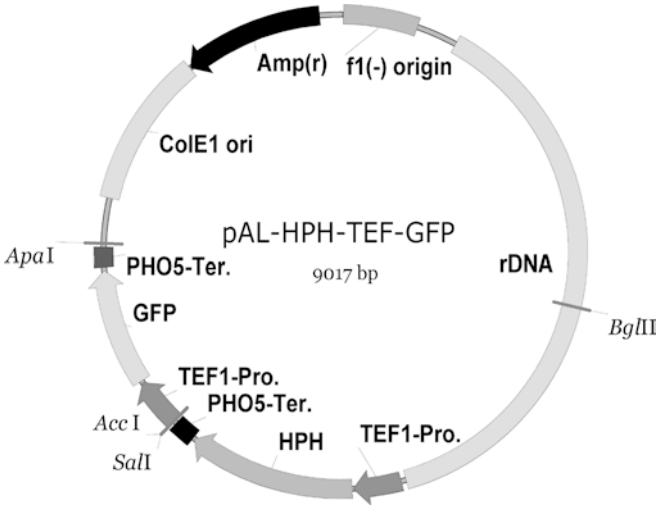

Fig. 1 Physical map of the expression/integration vector pAL-HPH-TEF-GFP used in this study. The vector contains the 25S rDNA sequence of *Arxula adeninivorans* (rDNA, *grey segment*) and an expression cassette for the *Escherichia coli*-derived *hph* gene in the order: *A. adeninivorans*-derived *TEF1* promoter (*TEF1-Pro.*, *black arrow*), the *hph*-coding sequence (*HPH*, *grey segment*), *Saccharomyces cerevisiae*-derived *PHO5*terminator (*PHO5-Ter.*, *black bar*) as selection marker. The vector further contains a second expression cassette with *TEF1* promoter-green fluorescent protein (*GFP*) ORF-*PHO5*terminator elements

protein (*GFP*) gene. The vector construction has been previously described by Wartmann et al. [38].

Protein analysis

SDS-PAGE (with 6 μg cell extract protein) and Western blots were performed as described by Kunze et al. [23]. The dye binding method of Bradford [2] was used for quantitative determination of protein concentration in cell extract with bovine serum albumin as a standard protein. GFP was immunologically detected by Western blot analysis using a specific anti-GFP antibody (anti-GFP rabbit serum, diluted 1:2,000, Molecular Probes, Eugene, Ore.) for detection. Blots were stained with Western Blue Stabilized Substrate (Promega, Mannheim, Germany).

Fluorescence microscopy

GFP expression was visualised in yeast cells by fluorescence microscopy (Axioskop, Zeiss, Jena, Germany; excitation at 470 nm and detection using the BP500–530 nm emission filter). These conditions allowed visualisation of GFP-mediated fluorescence avoiding a significant auto-fluorescence background. Single images (512×512 pixels) were collected using line-averaging eight times, each for 1 s.

Results

Genomic integration of the vector pAL-HPH-TEF-GFP in *A. adeninivorans*, *D. hansenii*, *D. polymorphus*, *H. polymorpha*, *S. cerevisiae* and *P. pastoris*

To demonstrate the functionality of the vector pAL-HPH1, an expression cassette encompassing a *GFP*reporter gene under the control of the strong constitutive *A. adeninivorans*-derived *TEF1* promoter and the *S. cerevisiae*-derived *PHO5* terminator was inserted. The resulting plasmid, pAL-HPH-TEF-GFP (Fig. 1), was linearised by *Bgl*II restriction and used to transform strains *A. adeninivorans* LS3, *A. adeninivorans* 135, *D. hansenii* H158, *D. polymorphus* H120, *H. polymorpha*RB11, *S. cerevisiae* C13ABYS86 and *P. pastoris* GS115.

Independent of the yeast species, transformation frequencies between 50 and 200 transformants per microgram linearised plasmid DNA were achieved. In contrast, transformation frequencies were very low (<10 transformants μg^{-1}), when circular plasmid DNA was employed. Transformants of all yeast species were inspected for heterologous DNA content by Southern hybridisation and by re-transformation of *E. coli* with the yeast DNA for identification of potentially present free plasmids. In no case were *E. coli* transformants obtained. This demonstrates that no free plasmids with an Amp^{r}selection marker and the *E. coli* ori sequence were present in the selected yeast transformants.

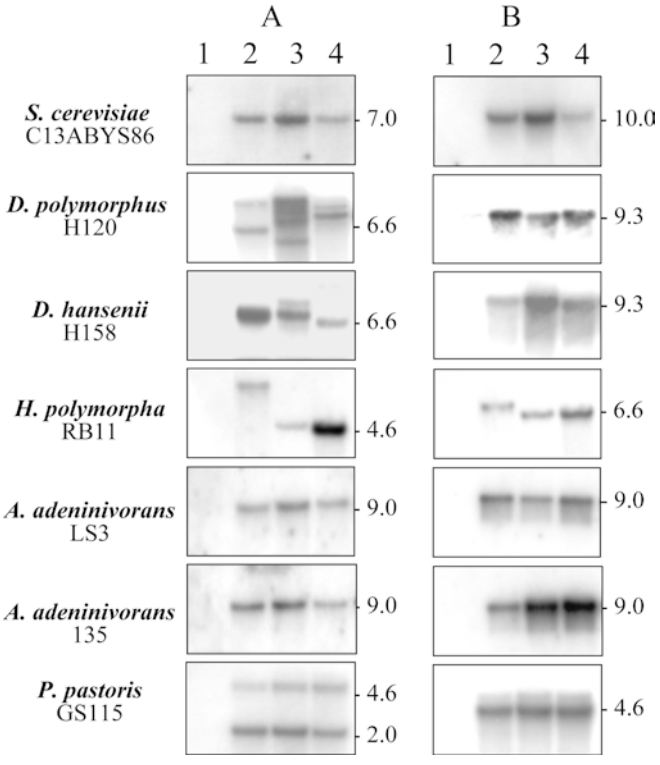

Fig. 2a, b Southern blot analysis of recombinant *A. adeninivorans*, *Debaryomyces hansenii*, *Debaryomyces polymorphus*, *Hansenula polymorpha*, *Pichia pastoris* and *S. cerevisiae*cultures. Genomic DNA was isolated as described in Materials and methods and restricted with *Acc*I (**a**) or *Bgl*II (**b**). Restricted DNA (1 μg) was separated on 0.8% agarose gels, transferred to nitrocellulose membranes and hybridised to a ^{32}P-labelled *GFP* gene probe. Lanes:*1* Untransformed strains, *2–4* transformants

To identify the integration site, genomic DNA was digested with *Acc*I (Fig. 2a) or *Bgl*II (Fig. 2b), which each restrict the plasmid pAL-HPH-TEF-GFP at a single site, and subjected to Southern blot analysis for the detection of integrated expression cassettes. An exact copy number determination was omitted, since no suitable internal marker was available for most of the species tested.

The budding cell-forming strain *A. adeninivorans* LS3, as well as the mycelia-forming strain 135, contained the *GFP* gene cassette on a ca. 9.0 kb *Acc*I/*Bgl*II fragment. Only a single hybridisation signal of similar intensity was detected in the various digests.

A similar situation was observed in *S. cerevisiae* transformants. Here, a hybridisation signal of either 7.0 kb (*Acc*I) or 10.0 kb (*Bgl*II) was visible, although of different signal intensities in the selected strains. In recombinant *D. polymorphus* strains H120/pAL-HPH-TEF-GFP two to four 4.6–9.0 kb *Acc*I DNA fragments were identified. Digestion with *Bgl*II, however, revealed a strong variable hybridisation signal at 9.3 kb, substantiating the possibility that some of the integrated plasmids might have a genomic location different from that of the rDNA locus.

All *P. pastoris* strains GS115/pAL-HPH-TEF-GFP showed two identical *Acc*I DNA fragments (4.6 and

2.0 kb) and only one *Bgl*II fragment of 4.6 kb with the *GFP* gene.

In *D. hansenii* H158/pAL-HPH-TEF-GFP, and *H. polymorpha*RB11/pAL-HPH-TEF-GFP, only a single hybridisation signal was detected. Again, a differential hybridisation pattern was observed. In *D. hansenii* H158, the *GFP* expression cassette is present on an *Acc*I fragment of about 6.6 kb, whereas the size of the *Acc*I fragments varied between 4.6 and 9.0 kb in *H. polymorpha* transformants, similar to the situation in *D. polymorphus* (Fig. 2).

GFP expression in the selected strains

For determination of heterologous gene expression in the various hosts, we selected the intracellular GFP as a reporter protein following the design of previous studies [38]. For this purpose, yeast transformants were cultured in YEPD medium at 30°C for 48 h and then qualitatively analysed for the presence of GFP by western blot analysis. In all transformants, GFP was present in reproducibly high concentrations. In contrast to the situation in *A. adeninivorans* and *P. pastoris* transformants, where only a single GFP species of 27 kDa was observed, an additional protein band of low intensity was immunologically detectable in protein extracts from *S. cerevisiae*, *D. polymorphus*, *D. hansenii* and *H. polymorpha* transformants. The GFP concentration was estimated by comparing the signal intensities derived from Western blot analysis of a single individual polyacrylamide gel. Among the various transformants, *A. adeninivorans* strains contain the strongest GFP signals followed by those of *S. cerevisiae* and then *D. polymorphus* transformants, with each group exhibiting a divergent extent of productivity by selected strains. The lowest GFP signals were observed in *D. hansenii*, *H. polymorpha* and *P. pastoris*. Again, the GFP signal intensity revealed titre differences of more than 5-fold among *D. hansenii*transformants (Fig. 3).

In a second approach, intracellular GFP accumulation was visualised by fluorescence microscopy. In all yeast transformants tested, GFP was distributed homogeneously within the cytoplasm and was completely excluded from vacuoles as expected from a GFP translation product without a secretion leader sequence (Fig. 4).

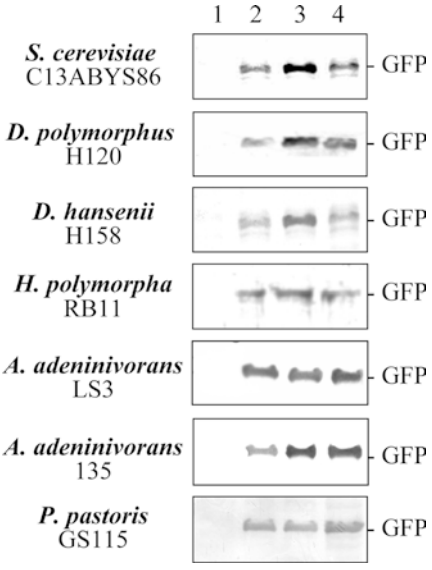

Fig. 3 Presence of GFP in recombinant *A. adeninivorans*, *D. hansenii*, *D. polymorphus*, *H. polymorpha*, *P. pastoris* and *S. cerevisiae* cell cultures. Yeast strains were cultured in YEPD medium for 48 h at 30°C. Cell extract samples were then prepared as described in Materials and methods. Cell extract proteins (6 μg) were separated on 12.5% SDS-polyacrylamide gels, transferred to nitrocellulose membranes and probed with anti-GFP antibodies. Lanes:*1* Untransformed strains, *2–4* transformants

Discussion

Yeast systems differ in their capability to produce a given foreign protein [9, 40]. Thus, it is desirable to pre-select the most suited expression platform from a range of candidates in a particular strain development. However, established systems, such as *S. cerevisiae*, *P. pastoris* or *H. polymorpha*, are based on species-specific vectors that work efficiently only in the homologous system [4, 9, 15]. A wide-range yeast vector should include a selection marker, a conserved targeting sequence, and a promoter that functions comparably in all yeasts to be tested.

The hygromycin selection marker based on the *E. coli*-derived *hph* gene has been successfully used in many yeast systems such as *S. cerevisiae*, *Trichosporon cutaneum* or *Candida oleophila* [12, 18, 41], the 25S rDNA provides a conserved repetitive DNA sequence suitable for integration [28], and the strong constitutive *A. adeninivorans*-derived *TEF1* promoter was found to be active in all yeast species analysed so far (unpublished results). These components were previously successfully combined on a basic vector to generate recombinant *A. adeninivorans* strains [30—32, 38]. Equipped with an easily detectable *GFP* reporter sequence, in this study this vector was applied to a broad spectrum of different yeasts, i.e. *A. adeninivorans*, *S. cerevisiae*, *D. hansenii*, *D. polymorphus*, *H. polymorpha* and *P. pastoris*. Exceeding our expectations, all yeast strains tested could be transformed, and GFP-producing recombinants could be generated. Detection of GFP served to confirm proof of concept, therefore precise quantification and standardisation of the GFP content in the various strains was not anticipated, and the obvious discrepancies in signal intensity in Southern and Western blot were not further pursued (Figs. 2 , 3).

In the recent past, rDNA has served as a target sequence for homologous recombination for a range of yeast systems, including *S. cerevisiae* [25], *K. lactis* [1], *Y. lipolytica* [24] and *H. polymorpha* [5]. In *H. polymorpha*, a single-step co-integration of multiple expression cassettes was even observed when using an

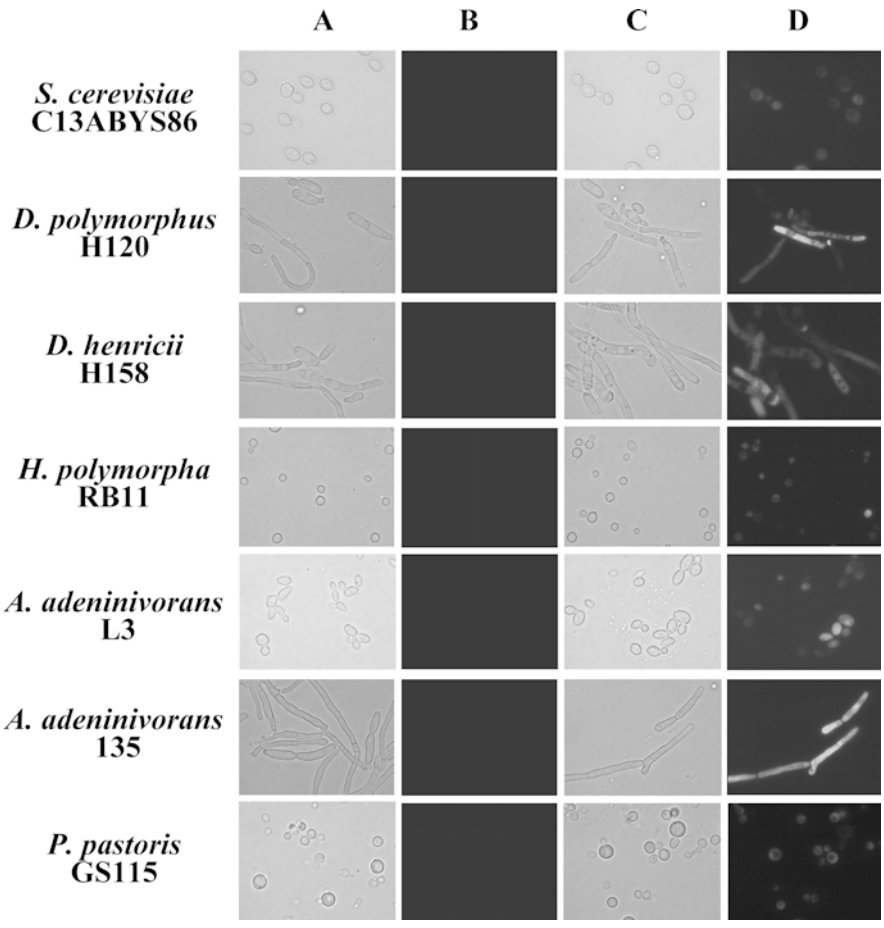

Fig. 4a–d Detection of recombinant GFP-producing yeast cells by fluorescence microscopy. Untransformed yeasts (**a, b**) and transformants (**c, d**) were cultured for 48 h in YEPD medium at 30°C and subsequently used for staining. **a, c** transmission; **b, d** GFP-fluorescence

rDNA integration fragment including 500 bp of the 25S 3′ end, and the 5S region flanked by NTS1 and part of NTS2 [19]. However, this vector could not be applied to other systems since the NTS2 end is not conserved among species, thus preventing homologous recombination into the genomes of heterologous yeasts. To achieve multi-host integration, an alternative targeting sequence had to be defined, now comprising the full-length 18S sequence, the ETS region and the NTS2 sequence including the putative promoter of the 35S precursor. This targeting sequence was included in a vector that could be successfully applied to generate recombinant *H. polymorpha*, *S. cerevisiae*, *P. stipitis* and *A. adeninivorans*strains [20]. In parallel, we assessed the *A. adeninivorans*-derived 25S rDNA fragment for integration in heterologous systems, as reported in the present study. Here, we see some evidence that the linearised vector is not exclusively targeted to the rDNA locus in heterologous organisms. The reason for this phenomenon has to be studied in more detail. Nevertheless, the 25S rDNA can be applied as targeting element for wide-range yeast integration vectors.

The results presented reveal the universality of the *A. adeninivorans*-derived *TEF*1 promoter, which elicits heterologous gene expression in all five yeast species tested in this study. Molecular analysis of this promoter showed that it contains typical elements of a yeast transcriptional promoter such as "CAAT" and "TATA" boxes [30].

In summary, the vector described in this study offers a convenient tool to create, in parallel, a range of recombinant yeast strains based on various species that enables a comparative assessment of such yeasts for the identification of an optimal host.

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