

Overexpression of the yeast *HAM1* gene prevents 6-*N*-hydroxylaminopurine mutagenesis in *Escherichia coli*[★]⊗

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The base analogue 6-*N*-hydroxylaminopurine (HAP) is a potent mutagen in a variety of prokaryotic and eukaryotic organisms. Mutations in the yeast *ham1* gene render the cells hypersensitive to the mutagenic effect of HAP. We have found that this gene has homologues in a variety of organisms from bacteria to man. We have overexpressed yeast *Ham1p* in *E. coli*. We demonstrate that under conditions when this protein constitutes approximately 30% of cellular protein, the host strain is protected both from toxic and mutagenic effects of HAP. This result indicates that sole *Ham1p* activity might be sufficient for destruction of HAP or its metabolites in bacterial cells.

Analogues of natural precursors of nucleic acids can exert strong toxic effects due to interference with cellular metabolism (see Kornberg & Baker, 1992) and may be strong mutagens inducing replication mistakes due to ambivalent pairing capacity (Freese, 1959). Some of them, such as 8-oxoguanine, may be formed *in vivo*, thus being a normal cellular

component affecting replication fidelity and requiring a special protective system (Michaels & Miller, 1992).

6-*N*-Hydroxylaminopurine (HAP, see Khromov-Borisov (1997) for justification of the use of chemical names of bases analogues and abbreviations accepted in our paper) is a potent base analogue mutagen effective both in

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Abbreviations: HA, hydroxylamine; HAP, 6-*N*-hydroxylaminopurine; HAPR, 6-*N*-hydroxylaminopurine riboside; AHAP, 2-amino-6-*N*-hydroxylaminopurine; HC, 4-*N*-hydroxylaminocytidine; AP, 2-aminopurine, MP, 6-mercaptapurine; MGD, molybdopterin guanine dinucleotide; Am, ampicillin; Rif, rifampicin; SDS/PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

eukaryotes and prokaryotes (for reviews see Pavlov *et al.*, 1991, Kozmin *et al.*, 1998). In an attempt to reveal the genes controlling HAP mutagenesis in yeast, we isolated the *ham1-1* mutant which was hypersensitive to HAP-induced mutagenesis (Pavlov, 1986). The observed hypersensitivity was HAP-specific, since no hypersensitivity to UV-light, ethylmethane sulfonate (EMS) and even to related base analogue, 2-amino-6-*N*-hydroxylaminopurine was found. It was demonstrated that the *ham1-1* or *ham1::LEU2* mutations did not influence spontaneous mutation and recombination rates. The *HAM1* gene was cloned, sequenced and its homologues were found in a variety of organisms (Noskov *et al.*, 1996; Kozmin *et al.*, 1998). We proposed that Ham1p might be involved in the HAP metabolism. We suggested that the major way of HAP utilisation is its deamination to nonmutagenic hypoxanthine, probably by adenine aminohydrolyase; however, neither *ham1-1* nor *ham1::LEU2* mutations had any influence on HAP deamination *in vivo* (Kozmin *et al.*, 1997). In present work we overexpressed *HAM1* gene in *E. coli* and studied its protective effects in the bacterial host.

MATERIALS AND METHODS

Plasmids. Plasmid pET-15b (Novagen Cat. No. 69257-1) was used as a vector for cloning and expression of the target yeast *HAM1* gene. The selective marker of pET-15b is ampicillin (Am). The plasmid pET-15b was cut at its unique *Bam*HI restriction site inside its cloning/expressing region, cut ends were Klenow filled-in and the resulting linear fragment was ligated with the 876 bp *Eco*RV/*Hpa*I fragment containing the *HAM1* gene of pFL44-HAM1 plasmid (Noskov *et al.*, 1996) (Fig. 1). The resulting plasmid was named pET-15b-HAM1.

Bacterial strains. *E. coli* strain XL1-Blue (Sambrook *et al.*, 1989) was used as a host for plasmid construction. Strain BL21(DE3)

(*ompT*, *hsdSB* (*rB*⁻ *mB*⁻), *gal*, *dcm* (DE3)) (Studier & Moffat, 1986) was used as a host for expression of the yeast Ham1p and also for studying the influence of Ham1p-overproduction on the sensitivity of bacteria to base analogues. Strain BL21(DE3) is lysogenic for DE3 phage, a lambda derivative carrying the gene for T7 RNA polymerase necessary for overexpression of the target *HAM1* gene linked with T7-promoter. Strains KA796 (*ara*, *thi*, Δ (*pro-lac*)) and NR10148 (*ara*, *thi*, Δ (*pro-lac*), Δ (*uvrB-bio*), *F'**pro-lac*) described earlier (Pavlov *et al.*, 1996), and MUT1 (*ara*, *thi*, *moeA*, *F'**pro-lac*) (Kozmin *et al.*, 1998) were used to test the inhibitory effect of base analogues.

Media. Luria broth (LB) was used as complete nutrient medium (Sambrook *et al.*, 1989). Complete medium with antibiotics contained 50 μ g/ml ampicillin (LB+Am) and 100 μ g/ml rifampicin (LB+Am+Rif). Minimal VB medium (Vogel & Bonner, 1956) contained 1 \times VB salts, 0.2% glucose, 5 μ g/ml thiamine and biotin and 50 μ g/ml proline.

Supplies. HAP was synthesized from 6-chloropurine according to the Giner-Sorolla & Bendich (1958). 2-Amino-6-*N*-hydroxylaminopurine (AHAP) and 4-*N*-hydroxylaminocytidine (HC) were a gift from Prof. C. Janion (Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Poland). 2-Aminopurine (AP), 6-*N*-hydroxylaminopurine riboside (HAPR), 6-mercaptopurine (MP) and hydroxylamine (HA) were from Sigma (U.S.A.). All these base analogues were dissolved in dimethylsulfoxide (DMSO) from Sigma. All reagents for SDS/PAGE were from Sigma. Restriction endonucleases and other DNA modifying enzymes were either from Fermentas (Lithuania) or New England Biolabs (U.S.A.).

SDS/PAGE. For sample preparation for SDS/PAGE, bacterial cultures were incubated overnight at 37°C with agitation in liquid LB+Am. After that 1 ml aliquots were centrifuged at 7000 \times *g* for 5 min at 4°C and the cell pellet resuspended in 100 μ l of ice-cold buffer, pH 8.0 (100 mM Tris, 1 mM EDTA,

Left arm of pET-15b *Bam*HI, Klenow Fill-in fragment

T7 promoter →

...CGAAATTAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAG
 AAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGGGCAGCAGCCATCATCATCAT
 MetGlySerSerHisHisHisHis
BamHI
 CATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGCTCGAGGAT-c
 HisHisSerSerGlyLeuValProArgGlySerHisMetLeuGluAsp G

+

Right arm of 876bp *Eco*RV/*Hpa*I fragment containing *HAMI* gene

*Eco*RV TUR of *HAMI* ... *HAMI* →
 AT-CTAAAACGAACGATAGCAGAATGAGCAACAAC...579b.p..TAG...
 TA LeuLysThrAsnAspSerArgMetSerAsnAsn...193a.a..End...

↓

Fusion construction pET15b-HAMI

His-Tag

...**ATG**GGCAGCAGCCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGC
 ...**Met** GlySerSerHisHisHisHisHisHisSerSerGlyLeuValProArgGlySer
TUR of *HAMI* *HAMI* →
 CATATGCTCGAGGATCATCTAAAACGAACGATAGCAGAATGAGCAACAAC...TAG...
 HisMetLeuGluAspHisLeuLysThrAsnAspSerArgMetSerAsnAsn...End...

Figure 1. Principle scheme of plasmid pET-15b-HAMI construction.

Underlined are gene parts corresponding to: → T7 promoter; — His-Tag sequence of pET-15b vector; --- Translated upstream region (TUR) of *HAMI*; → *HAMI* ORF.

0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10% glycerol). Suspended cells were disrupted by three cycles of deep freezing (-70°C) and thawing. The cell debris was sedimented by centrifugation for 10 min at 12000 × *g* at 4°C, and 50 μl aliquots of the supernatant were mixed with equal volumes of the buffer containing 0.75 mM Tris,

4% SDS, 5% mercaptoethanol and 20% glycerol (pH 6.8) and boiled for 2 min aliquots of 70 μl were loaded onto SDS/polyacrylamide gel. Electrophoresis was carried out in 10T2.7C gel by a standard method (see Laemmli, 1970).

Growth inhibition test 'A'. About 10⁸ cells of 6-10 independent bacterial clones were in-

oculated into 10 ml of liquid VB medium without inhibitors and with 0.1 mM of either HAP, HAPR, AHAP, HC, HA or 10 mM MP. The cultures were incubated for 19 h (strains KA796, NR10148, MUT1) or 24 h (strains BL21(DE3)-pET-15b and BL21(DE3)-pET-15b-HAM1) at 37°C with agitation. The absorbance of cultures was then measured at 600 nm. The values obtained for the given number of cultures in particular conditions were compared to those for other conditions by Wilcoxon non-parametric criterion.

Mutagenesis/growth inhibition test 'B' protocols. Bacterial transformation was performed as described by Mandel & Higa (1970).

BL21(DE3)-pET-15b or -pET-15b-HAM1 transformants (10 independent clones for each plasmid) were incubated for 24 h with agitation at 37°C in liquid LB+Am medium to stationary phase. About 10^4 cells from all 10 cultures (for mutagenesis test) or from 5 cultures (for growth inhibition test 'B') were then added to 10 ml of LB+Am liquid medium containing an appropriate amount of one of the base analogs (HAP, AHAP, AP, HC) or control solvent and grown for 24 h with agitation at 37°C. Aliquots of 100 μ l of bacterial culture diluted (4-fold for HC, 20-fold for AHAP and 10-fold for HAP; 5 μ g/ml in the variant of the test with the strain carrying pET-15b), concentrated (3-fold for 1 μ g/ml of HAP in the variant of the test with the strain carrying

pET-15b-HAM1) or original (for AP and 5 μ g/ml of HAP in the variant of the test with the strain carrying pET-15b-HAM1) were plated onto LB+Am+Rif plates for selection of Rif^r mutants. Viable counts were determined by plating aliquots of appropriately diluted cell suspension onto LB+Am plates. All plates were incubated overnight at 37°C. The frequency of Rif^r mutants was calculated for each of the 10 independent clones according to the formula: $F = (M \times a)/(N \times b)$ where "M" is the number of colonies on LB+Am+Rif plates, "N" the number of colonies on LB+Am plates, "a" and "b" – dilution factors. Frequencies were compared with the use of Wilcoxon non-parametric criterion.

RESULTS AND DISCUSSION

Overproduction of the HAM1p-fusion protein in pET-15b-HAM1 transformants of BL21 strain during growth under normal conditions (without isopropyl β -D-thiogalactopyranoside, IPTG, induction), was verified by SDS/PAGE. As shown in Fig. 2, strong band in lanes representing samples from pET-15b-HAM1 transformants corresponds to a protein with a molecular mass of about 30 kDa. The apparent molecular mass of this fusion protein is close to the expected mass of 29.8 kDa for the fusion protein (25.6 kDa for Ham1p plus 4.2

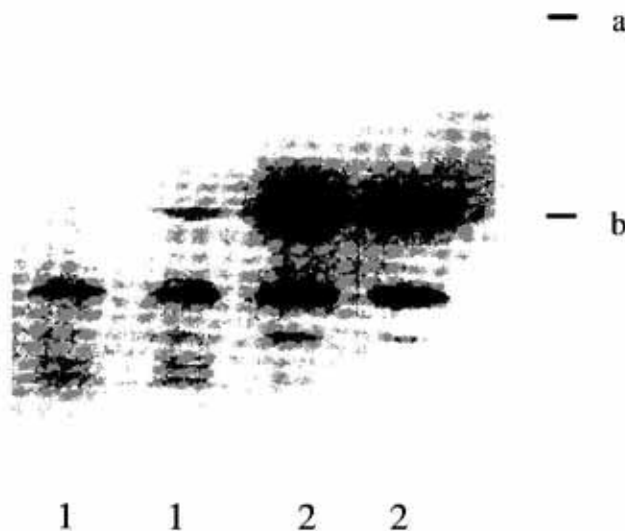


Figure 2. SDS/PAGE of total protein from strains BL21(DE3)-pET-15b (1) and BL21(DE3)-pET-15b-HAM1 (2).

Molecular mass markers: a, trypsin inhibitor (20 kDa); b, carbonic anhydrase (29 kDa).

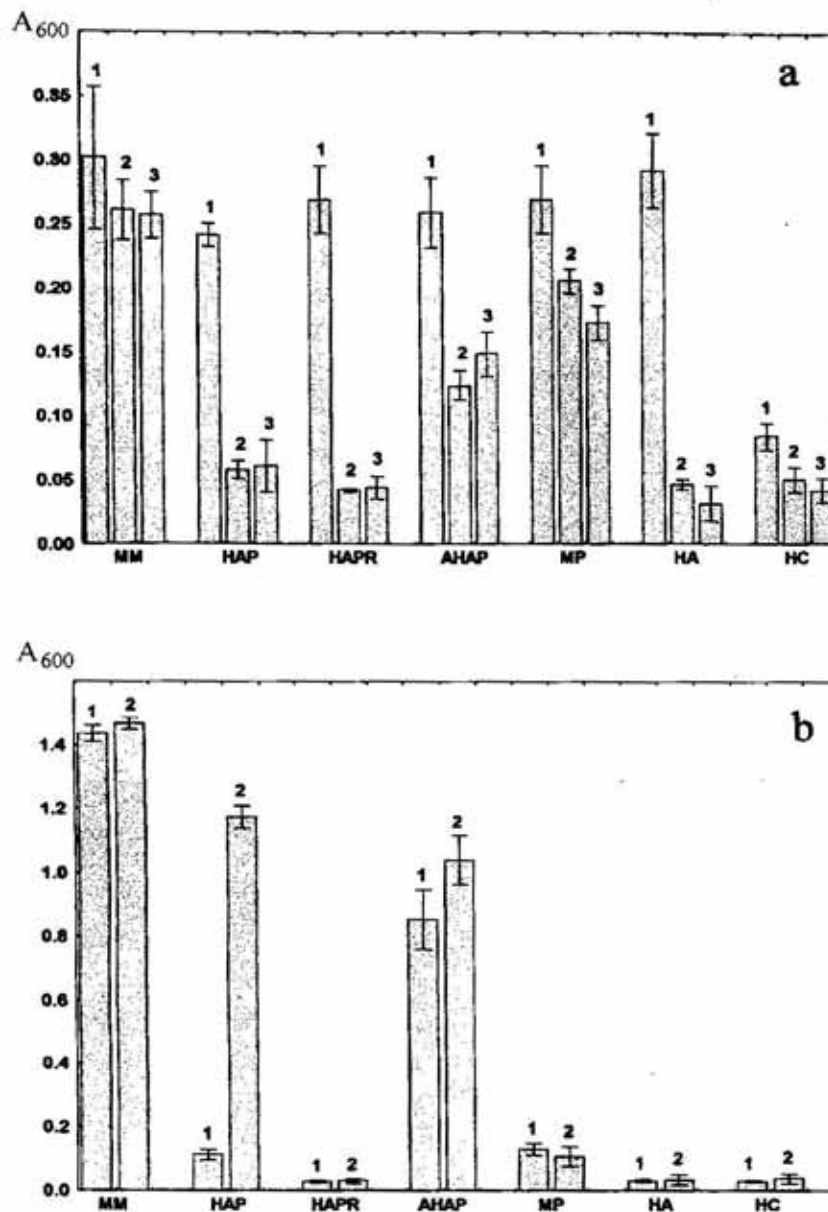


Figure 3. The inhibition of growth of bacterial strains by various analogues (test 'A').

a. Strains KA796 (1), NR-10148 $\Delta(wrb-bio)$ (2) and MUT1 (*moeA*) (3). Incubation 19 h at 37°C. **b.** Strains BL21-pET-15b (1) and BL21-pET-15b-HAM1 (2). Incubation 24 h at 37°C. Common notes: MM – minimal medium without analogues; HAP, HAPR, AHAP, HC, HA – the same medium with corresponding analogues added at 0.1 mM; MP – MM with 10 mM of mercaptopurine.

kDa of additional amino acids after fusion, see Fig. 1).

The next step of this study was to test if the yeast Ham1 fusion protein is functional in bacterial cell. At first we used a growth-inhibition test 'A' to examine the effect of HAP in the strain overproducing Ham1p and in the control strain (Fig. 3). The result of this test revealed high sensitivity to HAP of the BL21(DE3) strain due to a so far unknown reason (see discussion below). Overproduction of Ham1p in BL21 carrying pET-15b-HAM1 substantially suppressed the inhibitory effect of HAP. These data demonstrate

that yeast Ham1p functions in *E. coli* in a similar way as in yeast and suggests that this system may be used for studying the functions of Ham1p. It is known that bacterial cells are sensitive to the mutagenic or toxic effects of a substantially broader range of purine and pyrimidine analogues (like AP, HAPR, AHAP and HC) than yeast cells. This permits evaluation of Ham1p function with respect to the analogues that can be hardly tested in yeast. We studied the effects of these compounds with the use of tests for growth inhibition and in a fluctuation mutagenesis test.

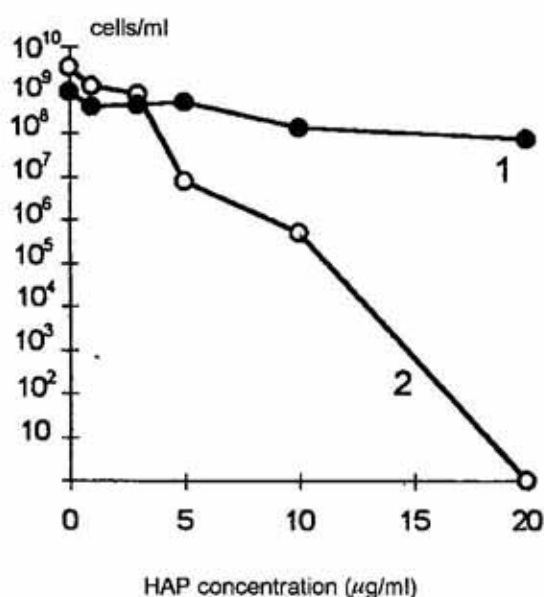


Figure 4. The inhibitory effect of HAP in liquid complete LB medium on bacterial growth for strains BL21(DE3)-pET-15b-HAM1 (1) and BL21(DE3)-pET-15b (2) (test 'B').

In the growth-inhibition test 'A' we used also some strains highly sensitive to hydroxylamine and some base analogues, including HAP. These strains carry either the 2 min-long deletion spanning *uvrB* and *bio* genes or a point mutation in the *moeA* gene of this region. They are defective in the synthesis of the molybdopterin guanine dinucleotide (MGD) due to inactivation of some molybdenum-dependent enzyme(s) that somehow protect(s) cells from HA and base analogues (sensitivity of *uvrB-bio* *Salmonella* strains was first no-

ticed by Janion (1979); see discussion of current status of this problem in Kozmin *et al.* (1998)). Results of these tests are shown in Fig. 3. Strains with a defect of MGD synthesis NR10148 and MUT1, show hypersensitivity to the toxic action of HA, HAP and HAPR and significantly higher sensitivity to AHAP, MP and HC as compared to the wild-type strain KA796. Strain BL21(DE3) has a phenotype similar to *Mgd*⁻ strains and we propose that this strain carries a mutation in one of the genes involved in MGD biosynthesis or in the gene for MGD-dependent enzyme that protects cells from analogues. Overproduction of Ham1p in strain BL21(DE3) led to strong suppression of hypersensitivity to HAP, weak suppression of sensitivity to AHAP and did not affect sensitivity to HAPR, HA, HC or MP. The toxic effect of compounds tested did not correlate with their mutagenic properties. The most mutagenic analogue AHAP was shown to exert only a weak toxic effect, while a comparatively poor mutagen HC exhibited the most profound toxic effect, even for the wild-type strain KA796 (see Fig. 3 and Table 1).

We tested the influence of overproduction of Ham1p on the mutagenic activity of HAP, AHAP, AP and HC (Table 1). Statistically significant differences in the levels of induced mutations between the control and Ham1p overproducing strain were observed only in the case of HAP. The increased level of

Table 1. AP, HAP, AHAP and HC induced mutant frequencies in the strain BL21(DE3)-pET-15b and BL21(DE3)-pET-15b-HAM1

Strain	Rifampicin-resistant mutants frequencies ($\times 10^{-7}$) induced by:				
	AP 250 µg/ml	HAP*		AHAP 1 µg/ml	HC 10 µg/ml
		1 µg/ml	5 µg/ml		
BL21(DE3)- pET-15b	13 (11-15)	45 (31-59)	438 (292-584)	2700 (1900-3500)	71 (59-83)
BL21(DE3)- pET-15b-HAM1	15 (10-20)	7 (5-9)	11 (4-17)	4100 (3300-4900)	84 (78-90)

Note: Median values with 95% confidence interval (below) are presented. Spontaneous Rif^r mutant frequencies for these strains were less than 10^{-8} .

*Differences between strains are significant by Wilcoxon criteria ($P < 0.01$).

Ham1p resulted in a 6-fold decrease of the mutagenic effect of HAP at the concentration of 1 $\mu\text{g/ml}$ and in a 40-fold decrease – at the concentration of 5 $\mu\text{g/ml}$.

To measure quantitatively the growth-inhibitory effect of HAP, we estimated the number of viable cells at the stationary phase of growth as a function of HAP concentration in liquid complete growth medium (test 'B'). As shown in Fig. 4, at HAP concentration of 5 $\mu\text{g/ml}$, the approximate difference in cell titers between two BL21(DE3) carrying either pET15b or pET15b-HAM1 is 340-fold, and at 10 $\mu\text{g/ml}$ the difference is 10^3 -fold. At 20 $\mu\text{g/ml}$, no growth of the control strain BL21(DE3) carrying pET-15b plasmid was observed, whereas the Ham1p-overproducing strain was growing in the presence of HAP only 7-fold less efficiently than in its absence.

Comparison of the mutagenic and toxic effect of HAP in these two strains may indicate that these effects are of different nature, since a weak mutagenic effect of HAP contrasts with a strong effect on cell viability. A similar conclusion has been drawn earlier for AHAP (Janion & Myszkowska, 1981). Thus, the mechanism of bacterial sensitivity to HAP and other analogues is completely different from that of yeast sensitivity to HAP, which could be ascribed mainly to the induction of recessive lethal mutations (Noskov *et al.*, 1996).

Yeast *ham1* mutants are specifically hypersensitive to HAP and are not sensitive to other analogues (Pavlov, 1986). Application of the heterologous expression system of HAM1 permitted us to broaden the spectrum of analogues which could be studied and we demonstrated that expression of yeast Ham1p protein in bacteria does not affect the mutagenic effects of AHAP, AP and HC and toxic effects of HAPR, HA, MP and HC. Thus the activity of Ham1p seems to be specific with respect to HAP.

Although, the toxic HAP action seems to be unlinked to its mutagenic properties in bacteria, Ham1p strongly protects cells from both

effects of HAP. Based on this observation we conclude that Ham1p is not involved in some kind of DNA repair of HAP-containing DNA. Since the toxic effect of HA is not smaller than the toxic effect of HAP we propose that Ham1p enzymatic activity is not linked to HAP deamination to HA and hypoxanthine, as in this case Ham1p would not be able to protect bacterial cells from toxic action of HAP. We assume that Ham1p may function as a HAP specific oxidoreductase that converts HAP to adenine, or as an aminotransferase that transfers bound hydroxylamine to a hypothetical co-substrate converting HAP to hypoxanthine and a nontoxic co-product. As overproduction of HAM1p does not lead to a decrease of the HAPR induced growth inhibition we may conclude that HAPR is not a substrate for Ham1p. It is surprising that Ham1p overproduction only weakly suppresses the toxic effect of AHAP and has no influence on AHAP induced mutagenesis. It is possible that AHAP is a very poor substrate for Ham1p and a potentially protective effect of Ham1p on the mutagenesis test can be observed because AHAP concentration used leads to a hypermutability effect and in this case detection of very small differences could be impossible.

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