The Escherichia coli protein YjjG is a house-cleaning nucleotidase in vivo

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Abstract
House-cleaning enzymes protect cells from the adverse effects of noncanonical metabolic chemical compounds. The Escherichia coli nucleotide phosphatase YjjG (B4374, JW4336) functions as a house-cleaning phosphatase in vivo. YjjG protects the cell against noncanonical pyrimidine derivatives such as 5-fluoro-2'-deoxyuridine (5-FdUridine), 5-fluorouridine, 5-fluoroorotic acid (5-FOA), 5-fluorouracil, and 5-aza-2'-deoxycytidine. YjjG prevents the incorporation of potentially mutagenic nucleotides into DNA as shown for 5-bromo-2'-deoxyuridine (BrdU). Its enzymatic activity in vitro towards noncanonical 5-fluoro-2'-deoxyuridine monophosphate (5-FdUMP) is higher than towards canonical thymidine monophosphate (dTMP). The closest homolog in humans, HDHD4, does not show a protective effect against noncanonical nucleotides, excluding an involvement of HDHD4 in resistance against noncanonical nucleotides used for cancer chemotherapy. The substrate spectrum of YjjG suggests that its in vivo substrates are noncanonical pyrimidine derivatives, which might also include oxidized nucleobases such as 5-formyluracil and 5-hydroxyuracil.

Introduction
Bacteria are constantly exposed to innumerable toxic compounds, both substances from their environment, as well as by-products of their own metabolism (Bessman et al., 1996; Galperin et al., 2006). Several protection mechanisms evolved to cope with this challenge and range from blocking the uptake (e.g. by the outer membrane of gram-negative bacteria), to export by specific transporters (e.g. ABC transporters), to specific inactivation of these compounds by dedicated enzymes. An especially noteworthy class of toxic substances are noncanonical nucleobase compounds as these can either inhibit DNA replication directly or lead to an elevated mutation rate (Kamiya, 2003). Enzymes recognizing these noncanonical nucleobase derivatives are 'house-cleaning' nucleotide phosphatases that act on noncanonical nucleotides and, in doing so, prevent their incorporation into DNA and RNA (Bessman et al., 1996; Galperin et al., 2006). The most extensively studied of these enzymes is the Escherichia coli protein MutT, which specifically dephosphorylates 8-oxo-GTP and thereby reduces the mutation rate ~100–3200-fold (Treffers et al., 1954; Bacon & Treffers, 1961; Cox & Yanofsky, 1969; Maki & Sekiguchi, 1992). Several nucleotide phosphatases have been identified that show activity against canonical nucleotides with a $K_m$ (Michaelis–Menten constant) in the millimolar range. These $K_m$ values are higher than expected for an in vivo function in canonical nucleotide metabolism. In addition, these enzymes do not show an obvious knock-out phenotype under normal laboratory conditions except for elevated mutation rates – these can be as highly elevated as for the mutT deletion (~100–3200-fold) or only slightly elevated (~2-fold) like for the mazG mutant (Zhang & Inouye, 2002). Galperin et al. (2006) hypothesized that many of these nucleotide hydrolases, as well as other hydrolases, function as house-cleaning enzymes in vivo. However, the identification of their house-cleaning substrates remains a challenge, which can only be solved by extensive screening.

Here, the results of a screen of conserved hypothetical gene deletion mutants with the toxic noncanonical nucleoside 5-fluoro-2'-deoxyuridine (5-FdUridine) are presented.

Materials and methods
Strains, gene cloning, and protein purification
Single-gene knockout mutants of the E. coli KEIO Collection (Baba et al., 2006) were tested for 5-FdUridine sensitivity.

[The rest of the text is not included as it seems to be part of the figure and not readable.]
For confirmation of the 5-FdUridine phenotype, the yjjG mutant was removed by Flp-recombinase-mediated recombination (Datsenko & Wanner, 2000).

For complementation of the phenotype, yjjG was amplified by PCR from genomic DNA (E. coli K-12 BW25113) and cloned into pBad24HA (pBad24 (Guzman et al., 1995) with an HA-tag added at its C-terminus) using EcoRI and HindIII (forward primer: AATT GAATTC ACC ATGAAAGTGGAAGCTTTTGTTCT; reverse primer: AATT AAGCTT AGT GGA CAT ACT GAG CTT GC). The resulting construct was named pBad24HA-yjjG.

HDHD4, the closest human homolog of YjjG (see below), was PCR amplified from plasmid DNA (kindly provided by E. Van Schaftingen) (Maliekel et al., 2006) and cloned into pBad24HA using NcoI and HindIII (forward primer: AATTCCACC ATG GGG CTG AGC CGC GTG C, reverse primer: AATT AAGCTT AGT GGA CAT ACT GAG CTT GC). The resulting construct was named pBad24HA-HDHD4.

For protein expression and purification, yjjG was amplified by PCR from genomic DNA and cloned into pETM30 (EMBL-Heidelberg, Protein Expression Facility) using NcoI and HindIII (forward primer: AATTCCACC ATG GGG CTG AGC CGC GTG C, reverse primer: AATT AAGCTT AGT GGA CAT ACT GAG CTT GC). The resulting construct was named pBad24HA-yjjG.

Agar-plate-based sensitivity screen

The sensitivity of E. coli single-gene deletion mutants (Baba et al., 2006) towards 5-FdUridine was tested using an agar-plate-based phenotyping assay. Selected gene mutants (supplementary Table S1) were arrayed onto a 384-well plate. Growth of mutant colonies was scored after overnight incubation. Liquid handling and transfer of mutant cultures were performed using a Biomek 2000 laboratory robot (Beckman–Coulter).

Nucleotide metabolism sensitivity tests

For confirmation of the 5-FdUridine phenotype, ΔyjjG and the wild type (BW25113) were transformed with pBad24HA and pBad24HA-yjjG. High overexpression [e.g., by induction with 0.2% (w/v) L-arabinose] of YjjG was found to be toxic. Rescue experiments with YjjG were performed relying on the basal expression levels of the pBad vector or induced with 0.01% (w/v) L-arabinose. Precultures were diluted to an OD600 nm of 0.01 with and without added 5-FdUridine, and the cell density was measured at OD600 nm at 1 h-intervals (ELx808Biotek Instruments, Friedrichshall). The measurement was performed in triplicate with 5-FdUridine concentrations ranging from 0 to 1000 μM.

The sensitivity of ΔyjjG towards different types of nucleobase and nucleotide derivatives was tested using untransformed strains. Additional rescue experiments with pBad24HA or pBad24HA-yjjG transformed strains were performed in the presence of 0.01% (w/v) L-arabinose.

In vivo 5-bromo-2′-deoxyuridine (BrdU) incorporation

The yjjG mutant strain and the wild-type (E. coli K-12 BW25113) strain were tested directly as well as transformed with pBad24HA (vector control) and pBad24HA-yjjG (YjjG expression plasmid). The strains were inoculated into LB medium at an OD600 nm of 0.05; for transformed strains, 0.2% L-arabinose was added to the medium. After 2 h of incubation at 37 °C, the cultures were checked for an equal OD600 nm and BrdU was added to the medium (10 μM for untransformed strains and varying concentrations for the transformed strains). The cells were incubated for 2.5 h at 37 °C and total DNA was isolated using standard procedures. The DNA concentrations were adjusted to equal levels. The DNA was denatured by incubation with 0.5 M NaOH for 20 min at 42 °C and 2 μl was spotted onto a Hybond-N+ membrane (Amersham Biosciences). After drying, the DNA was UV crosslinked (Stratalinker, Stratagene). BrdU incorporation was measured by Western Blotting and anti-BrdU-peroxidase (POD; 1:1000, Roche). Blocking, incubation, and washing were performed with 1% (w/v) bovine serum albumin (BSA) and 1% (w/v) milk powder in phosphate buffered saline (PBS). A chemiluminescence-based detection with an ECL substrate (Amersham Biosciences) was used.

In vitro enzymatic assay

Nucleotidase activity towards thymidine monophosphate (dTMP) and 5-fluoro-2′-deoxyuridine monophosphate (5-FdUMP) was measured in 400 μL reaction buffer (50 mM Tris/HCl, pH = 7.5; 5 mM MgCl2; 0.5 mM MnCl2) with 0.01 μg purified YjjG and substrates at concentrations of 0–1500 μM. After 20–30 min at 37 °C, the reaction was terminated and the free P1 concentration was measured using 100 μL Malachite reagent (Baykov et al., 1988).

Results and discussion

yjjG mutant shows a high sensitivity towards 5-FdUridine

In an effort to elucidate the function of uncharacterized or only generally characterized proteins, a range of phenotyping
assays were conducted with 177 E. coli gene deletion mutants (Baba et al., 2006) (supplementary Table S1). A screen for mutants with altered sensitivity towards 5-FdUridine (Fig. 2b) yielded one highly sensitive mutant: ΔyjjG (B4374, JW4336) (Fig. 1a).

5-FdUridine is a toxic noncanonical nucleoside, which is either phosphorylated to 5-fluoro-dUMP by thymidine kinase (Tdk) or cleaved to 5-fluorouracil by thymidine phosphorylease (DeoA; Fig. 4) (O’Donovan & Neuhard, 1970). 5-fluoro-dUMP inhibits thymydylate synthetase (Thy) and thus blocks DNA synthesis (Cohen et al., 1958; Santi et al., 1974). Additionally, 5-fluorouracil can be incorporated into RNA and can block protein translation (Wilkinson & Crummy, 1977; Kufe & Major, 1981). Finally, 5-fluorouracil can eventually be incorporated into DNA, and thus, may interfere with DNA metabolism (Herrick et al., 1982; Major et al., 1982).

The growth of ΔyjjG was already completely blocked at a concentration of 1 μM 5-FdUridine in the agar-plate-based assay, whereas the wild type and the remaining mutants were not affected. As the mutant strains of the systematic knockout library used for the screen retain the kanamycin-resistance cassette used for gene inactivation, this cassette was removed for the following assays by the procedure suggested by (Datsenko & Wanner, 2000). The observed sensitivity towards 5-FdUridine was clearly reproduced in a growth assay with a striking effect even at 1 μM 5-FdUridine (Fig. 1b). This phenotype was largely rescued by expressing YjjG from a plasmid – even at basal (noninduced) expression levels. Stronger induction of YjjG expression by addition of 0.01% L-arabinose adjusted the sensitivity to wild-type levels (Fig. 2c). However, note that high induction of YjjG (e.g. with 0.2% L-arabinose) was toxic for the cell, probably due to its general activity towards pyrimidine nucleotides (Proudfoot et al., 2004).

Recently, YjjG was found in an in vitro screen for proteins with phosphatase activity and shown to have activity against dTMP, uridine monophosphate (UMP), and deoxyuridine monophosphate (dUMP; Proudfoot et al., 2004; Kuznetsova et al., 2006). Based on this observation, a function of YjjG in canonical nucleotide metabolism was suggested, probably with involvement in regulatory pyrimidine nucleotide substrate cycles.

Kuznetsova et al. (2006) already suggested a potential detoxification function for YjjG (and related hydrolases), and (Galperin et al., 2006) hypothesized that many nucleotide phosphatases identified to act on canonical nucleotides are indeed ‘house-cleaning’ phosphatases. These enzymes prevent the incorporation of noncanonical nucleotides into the metabolism and thus protect the cell from toxic side effects and mutations. The most extensively studied of these enzymes is the E. coli protein MutT, which specifically dephosphorylates 8-oxo-GTP and thereby reduces the mutation rate by ~100–3200-fold (Treffers et al., 1954; Bacon & Treffers, 1961; Cox & Yanofsky, 1969; Maki & Sekiguchi, 1992). The present finding clearly implies that YjjG has a ‘house-cleaning’ function in vivo rather than a regulatory function in canonical nucleotide metabolism: the assays clearly show a protection from toxic 5-FdUridine even at a concentration of 1 μM; no growth inhibition of the mutant could be observed under standard conditions, pointing to a nonessential role of YjjG in canonical nucleotide metabolism.
Fig. 2. The YjjG mutant is sensitive to several noncanonical pyrimidine derivatives. (a) Several noncanonical nucleosides were tested for toxicity in growth curve assays. Tested conditions are indicated in the diagram legend as follows: wild type (WT, solid line) or yjjG mutant (yjjG, dashed line) grown in LB medium with varying concentrations (µM) of a noncanonical nucleoside analog or nucleobase (as given in the title of the diagrams). (b) Summary of structures and effects of tested noncanonical nucleosides. Strong (++), intermediate (+), and no effect (−) on the YjjG mutant. For comparison, pyrimidine derivative concentrations reducing growth to 50 percent (at 8 h time point) are given in parentheses (ΔyjjG/WT). (c) Rescue of ΔyjjG sensitivity phenotypes by YjjG expression from a plasmid. The relative OD at 600 nm (OD 600 nm) comparing cells grown in the presence and absence of noncanonical nucleobase derivative is shown. Wild type (WT) and yjjG mutant (ΔyjjG) carrying an empty vector or an YjjG (+YjjG) expression vector are compared (induction with 0.01% L-arabinose). Measurement was taken after a 360-min incubation time. Cells were exposed to 1 µM 5-FdUridine, 500 µM 5-FOA, 10 µM 5-fluorouracil (5-FU), 5 µM 5-fluorouridine, and 50 µM 5-aza-2′-deoxycytidine.
The YjjG mutant is sensitive to noncanonical pyrimidine derivatives

To assess the activity of YjjG further, several related nucleobase/nucleoside compounds were tested in an *in vivo* assay (Fig. 2a and b) and it was found that YjjG protects the cell from all 5-fluoropyrimidine derivatives tested: 5-fluorouracil is metabolized by the pyrimidine salvage pathway, 5-fluoroorotate (5-FOA) is an intermediate of the *de novo* synthesis of pyrimidines, and 5-fluorouridine is the ribonucleoside analog of 5-FdUridine. The sensitivity of the *yjjG* mutant to these compounds could be restored to wild-type levels by expressing YjjG from a plasmid (Fig. 2c).

All of these derivatives can be assumed to be eventually converted into monophosphorylated nucleotides, which are potential substrates of YjjG – 5-fluorouracil is, for example, converted to 5-FdUridine (Harbers *et al.*., 1959), and 5-FdUridine, 5-bromodeoxyuridine, and 5-iododeoxyuridine are all 5'-monophosphorylated by the deoxythymidine kinase of *E. coli* (Okazaki & Kornberg, 1964).

A higher sensitivity of the *yjjG* mutant towards these fluorinated compounds is found regardless of the way in which the derivatives are channelled into nucleotide metabolism, indicating a general function of YjjG that is not focused on either the salvage pathway or *de novo* synthesis. Interestingly, YjjG not only mediated resistance against these uridine derivatives but also against the cytidine nucleoside analog, 5-aza-2'-deoxycytidine. However, for this nucleoside, the relative growth difference between wildtype and *yjjG* mutant cells appeared to be lower under all the conditions tested (Fig. 2a and c).

Finally, BrdU and 5-iodo-2'-deoxyuridine at concentrations as high as 1 mM were not toxic for the cell, preventing the estimation of an effect of YjjG in this assay – for these compounds, a low toxicity and no significant increase of the mutation rate despite incorporation into DNA have been observed previously (Lorkiewicz & Szybalski, 1960; Rosner & Yagil, 1970). However, the activity of YjjG towards BrdU was shown in an *in vivo* DNA incorporation experiment (see below).

Taken together, YjjG shows a ‘house-cleaning’ function for noncanonical pyrimidine nucleotides but is not specific for a single nucleotide species.

**YjjG prevents incorporation of noncanonical nucleotides into DNA**

House-cleaning phosphatases are expected to eventually prevent the incorporation of noncanonical nucleotides into DNA, and thus avoid a detrimental increase of the mutation rate. To test a direct effect of YjjG on the incorporation of these nucleotide analogs into DNA, a BrdU incorporation assay was conducted (Fig. 3a). Whereas BrdU is readily incorporated into DNA of *yjjG* mutants, no incorporation could be seen in the wild-type background. Additionally, an overexpression of YjjG in the *yjjG* mutant was able to rescue the phenotype to a large extent.

**YjjG shows *in vitro* selectivity towards noncanonical 5-FdUMP**

To assess whether YjjG could have selectivity for a noncanonical nucleotide, the *in vitro* activity of YjjG was compared...
against dTMP and 5-FdUMP (Fig. 3b). A nucleotidase assay was conducted in a buffer containing 5 mM Mg\(^{2+}\) and 0.5 mM Mn\(^{2+}\) similar to the buffer used by Proudfoot et al. for their initial phosphatase screens (Proudfoot et al., 2004). In E. coli, the free Mg\(^{2+}\) concentration was estimated to be 1–5 mM (Lusk et al., 1968; Alatossava et al., 1985), whereas the Mn\(^{2+}\) concentration is considerably lower (~10 \(\mu\)M) (Outten & O’Halloran, 2001). Hence, the mixture of both cations was assumed to be closer to the in vivo situation than a buffer with a single cation (Proudfoot et al., 2004; Kuznetsova et al., 2006).

Under these in vitro conditions, YjjG showed a \(K_m\) of 2.14 ± 0.49 mM (\(k_{cat} = 27.3 ± 4.3\) s\(^{-1}\); \(k_{cat}/K_m = 1.3 \times 10^4\) M\(^{-1}\) s\(^{-1}\)) for dTMP and a \(K_m\) of 0.237 ± 0.074 mM (\(k_{cat} = 47.0 ± 4.2\) s\(^{-1}\); \(k_{cat}/K_m = 2.0 \times 10^5\) M\(^{-1}\) s\(^{-1}\)) for 5-FdUMP. As anticipated, the values measured for dTMP corresponded to the results obtained by (Proudfoot et al., 2004) and (Kuznetsova et al., 2006) when measured in the presence of Mg\(^{2+}\) as a bivalent cation. The ratio of the selectivity constants (\(k_{cat}/K_m\)) calculated for 5-FdUMP and dTMP was ~16. Thus, YjjG showed selectivity towards the noncanonical nucleotide 5-FdUMP, which might be the biochemical basis for the phenotypes observed in vivo.

However, with ~0.2 mM, the \(K_m\) of YjjG for 5-FdUMP is higher than expected for a bona fide ‘house-cleaning’ substrate – at least if noncanonical substrates, which are toxic or mutagenic at very low concentrations, are considered. MutT, for example, has a \(K_m\) of ~0.5 \(\mu\)M for 8-oxo-dGTP (Maki & Sekiguchi, 1992). On the contrary, other ‘house-cleaning’ enzymes have considerably higher \(K_m\) values. YniC shows a \(K_m\) of ~0.6 mM for its substrate 2-deoxyglucose-6P (Kuznetsova et al., 2006); however, its substrate 2-deoxyglucose-6P is found in the cell with up to 100 mM (Thompson, 1987). Finally, the human ABC transporter ABCC5 shows a difference in its in vivo substrate concentration and \(K_m\) which is comparable to YjjG. ABCC5 exports 5-FdUMP with a \(K_m\) of 1.1 mM, but confers resistance to 5-fluorouracil and 5-FdUMP at considerably lower concentrations in the lower micromolar range (Pratt et al., 2005). Thus, detoxification reactions with \(K_m\) values in the millimolar range can be sufficient to confer resistance at lower concentrations. In addition, it cannot be excluded that – due to different cation concentrations or substrate channelling – the actual kinetic values in vivo are different from the in vitro results.

**House-cleaning functions of YjjG’s homologs**

Most of the tested substrates are used as chemotherapeutic drugs in cancer therapy. Therefore, we wondered whether human homologs of YjjG can interfere with cancer therapy. The closest homolog of YjjG in human is HDHD4 (30% sequence identity, 42% sequence similarity). HDHD4 is the best human candidate gene for a true ortholog of YjjG.
because it is related to YjjG by a reciprocal best blast hit (with E values of 6e-12 and 3e-13) and it occurs in the same phylogenetic branch in the Pfam database (Bateman et al., 2004). Moreover, HDHD4 was classified as an ortholog of YjjG by the INPARANOID database, which stores information on eukaryotic orthologs (O’Brien et al., 2005).

HDHD4, although recently identified as a phosphatase involved in neuraminic acid metabolism (Maliekal et al., 2006), was hypothesized to possess in addition a moonlighting house-cleaning function. It was tested whether HDHD4 has the propensity to replace YjjG functionally in the yjjG mutant (Fig. 3c). However, no phenotypic complementation by HDHD4 was observed. This clearly indicates that YjjG and HDHD4, despite their close sequence similarity, have evolved different specificities. Thus, the interference of HDHD4 with noncanonical pyrimidine nucleoside-based cancer therapy is unlikely.

The closest sequence homolog of YjjG in yeast is SDT1 (24% sequence identity, 44% sequence similarity); however, SDT1 is not classified as a sequence ortholog by the MBGD database (Uchiyama, 2003). Interestingly, Nakaniishi and Sekimizu discovered a nucleotidase activity for SDT1 directed against UMP and CMP (Nakanishi & Sekimizu, 2002). Additionally, overexpression of SDT1 in yeast cells leads to hypersensitivity to 6-azauracil, 5-fluorouracil, and 5-fluorocytosine, implying a physiological role of SDT1 as a house-cleaning nucleotidase.

These two homologs of YjjG, HDHD4 and SDT1, show different physiological functions despite comparable sequence similarity. Both enzymes are classified by the Pfam database as ‘haloacid dehalogenase-like hydrolases (PF00702)’ (Bateman et al., 2004), a large group of hydrolytic enzymes with more than 6500 members. This group shows diverse hydrolytic specificities (Koonin & Tatusov, 1994; Aravind & Koonin, 1998). The present data suggest that sequence similarity alone is not sufficient to identify specific functions; the identification of true functional orthologs of YjjG in other species should therefore not rely solely on sequence similarity but needs to be supported by experimental data.

Concluding remarks

It was found that YjjG functions as an in vivo house-cleaning phosphatase for noncanonical pyrimidine nucleotides. A range of substrates that are recognized in vivo were identified, it was shown that YjjG prevents their incorporation into DNA, and it was found that YjjG has a higher enzymatic activity towards noncanonical nucleotides than towards canonical nucleotides in vitro. Fig. 4 illustrates the canonical pyrimidine metabolism, the entry points of noncanonical compounds, and the reactions presumably catalyzed by YjjG. However, the in vivo relevance of the substrates tested is not clear. Reactive oxygen/nitrogen species are thought to be the main sources of damage to DNA and DNA precursors (Kamiya, 2003): oxidation products of pyrimidine DNA precursors include 5-formyluracil, 5-hydroxyuracil, and thymine glycol. Although their evaluation in the in vivo assay used in this study was not feasible (5-formyluracil and 5-hydroxyuracil did not show a toxic effect even at a concentration of 1 mM; data not shown), monophosphorylated derivatives of these compounds are potential substrates of YjjG. A higher sensitivity towards H2O2, however, was not observed for the yjjG mutant either (data not shown). Apart from oxidative damage, YjjG could also directly protect bacteria from modified pyrimidine nucleotides acquired from environmental sources. Bacteria of the gut might have the propensity to modify the pharmacokinetics of chemotherapy drugs based on modified pyrimidine compounds. In summary, the present findings imply that YjjG, with its broad pyrimidine nucleotide activity spectrum, is a house-cleaning candidate for newly identified noncanonical pyrimidine nucleotides found in the environment or as by-products of metabolism.

An especially noteworthy feature of YjjG and its homolog SDT1 is their activity against nucleotide monophosphates. All previously described house-cleaning phosphatases act on nucleoside triphosphates. Thus, YjjG and probably SDT1 constitute a new functional group of house-cleaning phosphatases. This clearly supports the hypothesis that the importance of the house-cleaning function suggests that many undiscovered house-cleaning enzymes are ‘lurking among the unannotated ‘conserved hypothetical’ ORFs found in microbial genomes’ (Galperin et al., 2006).

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Authors contribution

B.T. and R.H. contributed equally to this work.

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**Supplementary material**

The following supplementary material is available for this article online:

**Table S1.** Gene deletion mutants tested for 5-FdUridine sensitivity.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1574-6968.2006.00646.x (This link will take you to the article abstract).

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