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# Molybdenum cofactor-dependent resistance to *N*-hydroxylated base analogs in *E. coli* is independent of MobA function

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## Abstract

Lack of molybdenum cofactor (MoCo) in *Escherichia coli* and related microorganisms was found to cause hypersensitivity to certain *N*-hydroxylated base analogs, such as HAP (6-*N*-hydroxylaminopurine). This observation has lead to a previous proposal that *E. coli* contains a molybdoenzyme capable of detoxifying such *N*-hydroxylated analogs. Here, we show that, unexpectedly, deletion of all known or putative molybdoenzymes in *E. coli* failed to reveal any base-analog sensitivity, suggesting that a novel type of MoCo-dependent activity is involved. Further, we establish that protection against the analogs does not require the common molybdopterin guanine-dinucleotide (MGD) form of the cofactor, but instead the guanosine monophosphate (GMP)-free version of MoCo (MPT) is sufficient.

## 1. Introduction

Modified nucleobases that mimic the normal DNA or RNA bases are traditionally referred to as base analogs [1]. Such analogs, when displaying mutagenic or inhibitory properties, have proven to be useful tools for studying fundamental cellular processes, including the metabolism of nucleic acids precursors or the precise mechanisms of DNA replication, repair, and mutagenesis. A particularly interesting group of mutagenic base analogs is comprised of *N*-hydroxylated derivatives of purines and pyrimidines [2]. Examples of these agents include 6-N-hydroxylaminopurine (HAP), 2-amino-6-hydroxyaminopurine (AHAP), and $N^4$-hydroxycytidine. Notably HAP, an adenine analog in which the exocyclic amino group is replaced by a hydroxylamino group, has been found to be a highly effective mutagen in bacteria, yeast, and mammalian cells [3-5].

Studies of the genetic control of HAP-induced mutagenesis in *Saccharomyces cerevisiae* and *Escherichia coli* have led to identification of HAP-hypersensitive mutants, which are useful for understanding how cells may try to protect themselves against these agents. In yeast, drastically enhanced sensitivity to the mutagenic and toxic action of HAP was observed in *ham1* mutants [6,7]. Ham1p is a specific pyrophosphatase that hydrolyzes HAP deoxyribonucleoside triphosphate to the corresponding mononucleotide, thus preventing incorporation of the analog into DNA [4,8]. While *E. coli* contains a homolog for the *HAM1* gene, named *rdgB* [9,10], this system apparently plays a back-up role in HAP detoxification

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[11,12]. Its main function is likely the removal of (d)ITP or (d)XTP from the cellular (d)NTP pools [10-12].

In *E. coli* (and *Salmonella*), hypersensitivity to *N*-hydroxylated compounds, including hydroxylamine (HA), was found associated with a deletion of the chromosomal *uvrB-bio* region [13-16]. Subsequently, it was found that the hypersensitivity of the *E. coli* Δ(*uvrB-bio*) strains is not due to their *uvr* excision-repair defect but to their defect in synthesis of the molybdenum cofactor (MoCo) [17]. MoCo is an essential cofactor for a broad, heterogeneous group of enzymes present from bacteria to man that are involved in a variety of oxidation-reduction reactions [18,19]. Based on this finding, we proposed that in *E. coli* there exists a hitherto undescribed MoCo-dependent activity that acts to detoxify HAP and related compounds [17].

In the present investigation, we have attempted to identify the nature of the proposed molybdoactivity by deletion analysis of all known and hypothetical *E. coli* molybdoenzymes as identified by database searches (18 total). Interestingly this analysis, which included several strains containing multiple deficiencies, did not yield any base-analog sensitive strains. Secondly, we found that protection against the analogs does not depend on the common molybdopterin guanine-dinucleotide (MGD) form of the cofactor. Instead, the GMP-free version of MoCo (MPT) appears sufficient. Thus, a novel type of MoCo-dependent activity may be responsible for this type of base-analog resistance of *E. coli*.

## 2. Materials and methods

### 2.1 E. coli strains used in this study

All tests for base-analog sensitivity were performed in the NR10836 (*ara thi* Δ*prolac* F'*prolac*) background [17]. Except where indicated, the gene deletions used in this study were generated initially in the strain BW25113 (*lacI*$^{q}$ *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$) by the PCR-mediated gene replacement method of Datsenko and Wanner [20]. All experimental conditions, including the PCR conditions, used were as in [20]. Subsequently, the deletions were transferred to NR10836 by P1 transduction and selection for kanamycin or tetracycline resistance. P1 transductions were performed using P1*virA* as described [21]. The PCR primers used contained 50-nt extensions complementary to the left or right end of the gene to be deleted as well as a 20-nt priming 3' end complementary to either the kanamycin-resistance (Kan$^{r}$) module of plasmid pKD13 [20] or the *tetA tetR* tetracycline-resistance (Tet$^{r}$) module of transposon Tn*10*. The genomic base-pair coordinates (*E. coli* K12 MG1655, GenBank record U00096) [22] of the left and right endpoints of the deletions were: Δ*moaE*::*kan* - 818,560/818,961; Δ*moeB*::*kan* - 863,610/864,301; Δ*mogA*::*kan* - 9,300/9,845; Δ*mobA*::*kan* - 4,039,463/4,039,994; Δ*dmsA*::*kan* - 940,295/942,576; Δ*narG*::*tet* - 1,279,089/1,282,790; Δ*narZ*::*kan* - 1,536,929/1,540,580; Δ*napA*::*kan* - 2,298,338/2,300,719; Δ*torA*::*tet* - 1,058,510/1,060,971; Δ*torZ*::*kan* - 1,952,629/1,955,000; Δ*bisC*::*kan* - 3,712,184/3,714,265; Δ*fdnG*::*kan* - 1,545,465/1,548,426; Δ*fdoG*::*tet* - 4,080,844/4,083,805; Δ*fdhF*::*kan* - 4,295,275/4,297,356; Δ*ydeP*::*kan* - 1,582,267/1,584,508; Δ*ynfE*::*kan* - 1,656,169/1,658,477; Δ*ynfF*::*kan* - 1,658,596/1,660,957; Δ*ydhV*::*kan* - 1,749,803/1,751,824; Δ*yedY*::*kan* - 2,037,504/2,038,465; Δ*yagR*::*kan* - 298,003/300,114; Δ*selAB*::*kan* - 3,756,090/3,759,271; and Δ*selD*::*tet* - 1,845,037/1,846,018. The correctness of each deletion was confirmed in all cases by PCR amplification. Transposon insertions *moaA*::mini-Tn*10cam*, *moeA121*::mini-Tn*10cam*, and *xdhD*::mini-Tn*10cam* were obtained in NR10836 by random transposon insertion mutagenesis [17]. A multiply-deficient *mobA xdhA xdhD yagR ydhV yedY* derivative of NR10836 was constructed as follows. First, the *xdhD*::mini-Tn*10cam* derivative of NR10836 was made Δ*xdhA*::*kan* by P1 transduction using as donor strain K4633-Δ*xdhA*::*kan* obtained from L. Reitzer [23]. The kan$^{r}$ marker was eliminated from this strain using plasmid pCP20 [20], which induces induction of recombination between the

repeats flanking the *kan* gene. Similarly, the Δ*yagR*, Δ*mobA*, Δ*ydhV*, and Δ*yedY*::*kan* markers were added sequentially from the BW25113 background (see above) by P1 transduction followed by elimination of the kan$^r$ module using pCP20 as necessary.

### 2.2 Media and chemicals

Bacteria were grown in Luria-Bertani broth (LB) [21] or minimal glucose medium containing Vogel-Bonner salts [24]. Cultures were grown at 37°, except in the experiments using plasmid pCP20 (see above). Strains containing pCP20 were grown at 30°, but were switched to 42° when elimination of the plasmid was desired. HAP was purchased from ICN Biochemicals; 2-amino-HAP (AHAP) was synthesized from 2-amino-6-chloropurine by I. Kuchuk (Indiana University) according to a published method [25]. All other chemicals were purchased from Sigma-Aldrich.

### 2.3 Sensitivity tests

Spot tests for base analog sensitivity were conducted as follows. *E. coli* cultures (1 or 2 ml) were grown overnight in LB and were then diluted 30-fold in 10 ml of 0.9% NaCl. Small aliquots were then transferred to a minimal-medium plate using a 150-prong replicator device, as described [17]. Each prong transfers approximately 1-2 μl to the plate. After the spots had dried, a small aliquot (about 5 μl) of HAP or AHAP (50 μg in DMSO) or hydroxylamine (150 μg in water) was spotted onto the center of the plate. The plates were incubated at 37°C and inspected the next day for a zone of inhibition.

Chlorate resistance was determined by plating 50 μl of a $10^{-5}$ dilution of an overnight culture (~ 500 cells) on LB plates containing 0.2% $KClO_3$, which were then incubated overnight at 37° anaerobically using a Becton Dickinson BBL gas pack anaerobic system, as described [17]. Under these conditions, chlorate-sensitive strains do not form colonies, whereas chlorate-resistant strains plate with essentially 100% efficiency. Chlorate sensitivity is due to the activity of certain MGD-requiring molybdoactivities, such as nitrate reductase or trimethylamine-N-oxide reductase, which convert chlorate to toxic chlorite, and strains lacking MGD are consequently resistant [36].

## 3. Results

### 3.1 Investigation of known and putative E. coli molybdoenzymes

As a first approach to identifying the MoCo-dependent activity responsible for base-analog detoxification, we undertook an analysis of all known and putative *E. coli* molybdoenzymes. Based on amino-acid sequence similarities and structural information, including the nature of the catalytic site, MoCo-containing enzymes have been generally divided into 4 families: the dimethylsulfoxide (DMSO) reductase family, the sulfite oxidase family, the xanthine oxidase family, and aldehyde ferredoxin oxidoreductase (AFOR) family [18,19] (Table 1). Members of the last family are actually tungstoenzymes, containing a tungsten atom instead of molybdenum as part of the otherwise identical pterin cofactor [18,19]. Currently, all *E. coli* molybdoenzymes with an established metabolic function are members of the DMSO reductase family. As listed in Table 1, these members include DMSO reductase, two TMAO (trimethylamine-*N*-oxide) reductases (TOR-A and TOR-Z), three nitrate reductases (NAR-A, NAR-Z and NAP), three formate dehydrogenases (FDH-N, FDH-F and FDH-O) and biotin sulfoxide reductase [26,27]. In addition, sequence homology searches using TBLASTN (NCBI) and data present in the NCBI Conserved Domain Database [28] indicate the presence in *E. coli* of three additional putative members of the DMSO reductase family, YdeP, YnfE, and YnfF (Table 1). The latter two have been identified as alternative DMSO reductases [29]. The database searches also identified one putative member of the sulfite oxidase family, YedY

[30], one putative member of the aldehyde ferredoxin oxidoreductase family, YdhV, and three putative xanthine oxidases, XdhA, XdhD, and YagR [23].

Each of the genes encoding the indicated known or putative molybdoactivities listed in Table 1 were deleted, individually, as described in the Materials and Methods. The mutations were transferred to strain NR10836 [17] and tested for sensitivity to HAP as described previously [17]. Typically, strains deficient in MoCo biosynthesis display a wide zone of inhibition or inactivation (~ 50 mm in diameter) (see also Fig. 1A), whereas a wild-type strain is fully resistant (no inhibition zone). Interestingly, we found that none of the deletions listed in Table 1 conferred a HAP-sensitive phenotype. Inactivation of the putative xanthine oxidases, XdhA, XdhD, and YagR, were of particular interest because mammalian xanthine oxidase has been shown to be able to catalyze the *in vitro* reduction of HAP to adenine [31], which would constitute one possible way of HAP detoxification [17]. However, even the *xdhA xdhD yagR* triple mutation did not increase HAP sensitivity, as also reported previously [11]. The three *E. coli* xanthine oxidases are considered largely cryptic, although some low-level activity in a purine catabolic pathway has been described [23]. Thus, based on this survey of known and putative molybdoenzymes, the nature of the putative HAP-detoxifying activity remains still unidentified.

### 3.2 Selenoenzymes

Database searches for putative molybdoenzymes based on the *E. coli* genomic DNA sequence have as a potential limitation that UGA stop codons may encode selenocysteine in certain sequence contexts [32]. Therefore, it is possible that searches, due to the presence of the UGA codon, may miss certain selenoenzymes. We noted, for example, that the *E. coli* formate dehydrogenases, which are molybdoenzymes (see Table 1), are established selenoproteins [33]. Thus, we constructed *E. coli* strains defective in either selenocysteine biosynthesis (*selA* or *selD*) or selenocysteine incorporation (*selB*). The results showed that neither a Δ*selAB*::*kan* deletion or a Δ*selD*::*tet* deletion affected the sensitivity to HAP, AHAP or hydroxylamine (data not shown).

### 3.3 Analysis of genes involved in MoCo biosynthesis

As a further effort to understand the role of MoCo in HAP detoxification, we analyzed the individual steps in the biosynthesis of the cofactor itself. MoCo biosynthesis proceeds in a number of discrete steps [see 34 or 35 for reviews]. The final product in *E. coli* is MGD (Molybdopterin Guanine Dinucleotide), in which molybdopterin is connected by dinucleotide linkage to a GMP residue [34,35]. With few exceptions (see below), the known *E. coli* molybdoenzymes use this MGD form of MoCo. Previously, we demonstrated that *moeA*, *moaA*, and *modC* mutants, each defective in a different step of MoCo biosynthesis, yielded HAP hypersensitivity [17]. Here, we systematically inactivated genes in each of the defined steps of MoCo biosynthesis and tested the strains for HAP sensitivity. The biosynthesis scheme is outlined in Fig. 1A, which also shows the results of the HAP sensitivity tests. A defect in the *moaA* gene blocks the first step, the formation of the pterin derivative precursor Z by a cyclization reaction from GTP. Inactivation of the *moaE* or *moeB* genes prevents conversion of precursor Z to a dithiolene derivative named molybdopterin (MPT). Inactivation of the *mogA* and *moeA* genes blocks, respectively, activation and insertion of the molybdate into the MPT. Finally, inactivation of the *mobA* gene blocks the addition of GMP to MPT, the final step that leads to Molybdopterin Guanine Dinucleotide (MGD).

The results of the HAP sensitivity tests (Fig. 1A and Table 2) indicate that mutations blocking the synthesis of precursor Z or MPT or blocking activation and insertion of the molybdenum ion into MPT lead to HAP-hypersensitivity. The same mutations also increased sensitivity to the toxic effect of AHAP and hydroxylamine (HA) (Table 2) and conferred AHAP-

hypermutability (data not shown), consistent with our previous observations with the *moeA* mutant [17]. Interestingly, deletion of *mobA* did not lead to any sensitivity to HAP, AHAP, or HA (Fig. 1A,Table 2). The Δ*mobA* strain was, at the same time, clearly deficient in several other MoCo-dependent activities that require MGD, such as nitrate reductase or TMAO reductase (results not shown). The strain was also chlorate-resistant under anaerobic conditions (Fig. 1B), an established hallmark of MGD deficiency [36]. Chlorate resistance results from lack of certain MGD-containing enzymes, such as nitrate reductases, which convert chlorate into lethal chlorite [36].

Thus, our results indicate, unexpectedly, that the MoCo-dependent base-analog detoxification system does not require the MGD form of MoCo. Instead, the MPT form is presumably sufficient.

## 4. Discussion

The present results cast a new light on the mechanism(s) by which *E. coli* and related organisms are able to protect themselves against the effects of HAP and other *N*-hydroxylated base analogs. First, our analysis of the known and putative molybdoenzymes of *E. coli* from four separate gene families failed to identify the gene encoding the proposed MoCo-dependent HAP detoxifying activity. This is a noteworthy observation, as it suggests that a novel mechanism may underlie bacterial resistance. It cannot be excluded that our database searches have missed one or more members of the established families of molybdoenzymes. However, in this case one must assume that such members have significantly diverged amino acid sequences and may, in fact, belong to a previously unrecognized family.

Secondly, our experiments showed, unexpectedly, that HAP resistance is independent of the *mobA* gene and, hence, does not require the MGD form of molybdenum cofactor, the common form in most bacterial species. Of the molybdoenzymes in Table 1, members of the DMSO reductase family all require the MGD version of cofactor, while the other three families utilize the MPT form [18,19]. *E. coli* contains only a few, largely putative, members of the last three families: YdhV, YedY, and the three putative xanthine dehydrogenases XdhA, XdhD, and YagR (Table 1). Among these, the crystal structure of YedY has indeed been shown the presence of MPT as cofactor, but the activity and cellular function of the protein are unclear at this time [30,37]. The three putative xanthine oxidases may play a role in purine catabolism, although their main function remains to be determined [23]. Regardless of their functions, our experiments have shown clearly that none of these MPT-bearing enzymes play a role in HAP detoxification.

Thirdly, the fact that the large majority of the *E. coli* molybdoenzymes requires MGD (i.e., are *mobA* dependent) provided an opportunity to create a strain (*mobA, ydhV, yedY, xdhA, xdhD, yagR*, see Materials and Methods) that is effectively deficient in all activities listed in Table 1. Importantly, this strain proved still fully HAP resistant (not shown). This result effectively eliminates any possibility of overlapping (redundant) activities among the investigated genes being responsible for HAP detoxification.

It is interesting to note that, as far as we know, a specific metabolic function for MPT-requiring enzymes has been identified in bacterial systems in only two reported instances, both involving xanthine dehydrogenase activity: *Rhodobacter capsulatus* [38] and *Pseudomonas aeruginosa* [39]. Thus, our observation of the *mobA* independence of resistance to HAP and related *N*-hydroxylated base analogs represents a novel case.

We are currently engaged in a direct search for base-analog sensitive mutants using random mutagenesis to possibly identify the responsible gene(s). As an alternative, we are also

considering the possibility that the requirement for MTP may be unrelated to its role as enzyme cofactor. Such an activity has not been reported and remains speculative.

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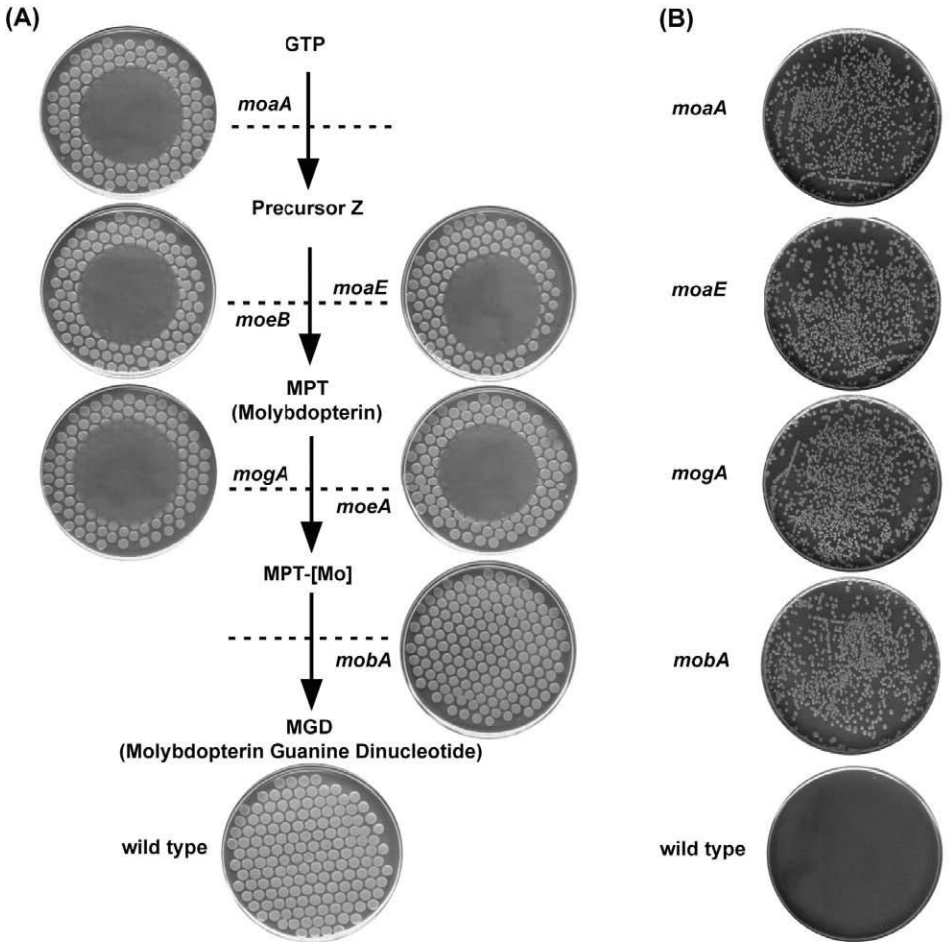


**Fig 1.**

(A) MoCo biosynthesis in *E. coli* and HAP sensitivity of strains blocked in the indicated steps in the MGD synthesis pathway. Deletions of indicated genes were created and transferred to strain NR10836 [17] as indicated in text. 50 μg of HAP was spotted onto the center of each plate as described [17]. (B) Chlorate resistance of the MoCo-deficient strains (see Materials and Methods). The combined (A) and (B) demonstrate that a *mobA* mutant is resistant to HAP while being defective in MGD evidenced by its chlorate resistance.

**Table 1**

Known and putative *E. coli* molybdoenzymes tested for HAP sensitivity

| Family of molybdoenzymes | Known or putative enzyme | Tested strain[a] |
|---|---|---|
| DMSO reductase family | DMSO reductase | Δ*dmsA*::*kan* |
| | Nitrate reductase A | Δ*narG*::*tet* |
| | Nitrate reductase Z | Δ*narZ*::*kan* |
| | Periplasmic nitrate reductase (NAP) | Δ*napA*::*kan* |
| | TMAO reductase A | Δ*torA*::*tet* |
| | TMAO reductase Z | Δ*torZ*::*kan* |
| | Biotin sulfoxide reductase | Δ*bisC*::*kan* |
| | Formate dehydrogenase N | Δ*fdnG*::*kan* |
| | Formate dehydrogenase O | Δ*fdoG*::*tet* |
| | Formate dehydrogenase H | Δ*fdhF*::*kan* |
| | Putative family members | Δ*ydeP*::*kan* |
| | | Δ*ynfE*::*kan* |
| | | Δ*ynfF*::*kan* |
| Aldehyde ferredoxin oxidoreductase family | Putative family member | Δ*ydhV*::*kan* |
| Sulfite oxidase family | Putative family member | Δ*yedY*::*kan* |
| Xanthine oxidase family | Putative family members | Δ*xdhA*::*kan* |
| | | *xdhD*::mini-Tn*10cam* |
| | | Δ*yagR*::*kan* |

[a] All strains were derivatives of NR10836 [17] created as described in Text.

## Table 2

Zones of inhibition produced by HAP, AHAP, and hydroxylamine (HA), measured by spot tests, for various strains affected in MoCo biosynthesis[a]

| Relevant genotype[a] | Diameter of the inhibitory zone (millimeters)[b] | | |
|---|---|---|---|
| | 50 µg of HAP | 50 µg of AHAP | 150 µg of HA |
| wild-type | 0 | 37 | 30 |
| *moeA* | 50 | 42 | 45/58[d] |
| Δ*mobA*::*kan* | 0 | 36 | 30 |

[a] All strains are derivatives of NR10836 [17].

[b] The numbers are an average for 5-10 experiments.

[c] Similar results were observed with other MPT-deficient mutants, such as *moaA*, *moeB*, *moaE*, and *mogA*; see also Fig. 1A.

[d] A secondary ring of inhibition (Ø = 58 mm) was noted in addition to the primary zone of inhibition (Ø = 45 mm)