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***YcbX* and *yiiM*, two novel determinants for resistance of *E. coli* to *N*-hydroxylated base analogs**

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Summary

We have shown previously that lack of molybdenum cofactor (MoCo) in *Escherichia coli* leads to hypersensitivity to the mutagenic and toxic effects of *N*-hydroxylated base analogs, such as 6-*N*-hydroxylaminopurine (HAP). However, the nature of the MoCo-dependent mechanism is unknown, as inactivation of all known and putative *E. coli* molybdoenzymes does not produce any sensitivity. Presently, we report on the isolation and characterization of two novel HAP-hypersensitive mutants carrying defects in the *ycbX* or *yiiM* open reading frames. Genetic analysis suggests that the two genes operate within the MoCo-dependent pathway. In the absence of the *ycbX*- and *yiiM*-dependent pathways, biotin sulfoxide reductase (BisC) plays also a role in the detoxification pathway. YcbX and YiiM are hypothetical members of the MOSC protein superfamily, which contain the C-terminal domain (MOSC) of the eukaryotic MoCo sulfurases. However, deletion of *ycbX* or *yiiM* did not affect the activity of human xanthine dehydrogenase expressed in *E. coli*, suggesting that the role of YcbX and YiiM proteins is not related to MoCo sulfuration. Instead, YcbX and YiiM may represent novel MoCo-dependent enzymatic activities. We also demonstrate that the MoCo/YcbX/YiiM-dependent detoxification of HAP proceeds by reduction to adenine.

Introduction

Modified nucleobases that can participate in cellular processes alongside the natural substrates but are toxic and/or mutagenic are traditionally referred to as base analogs (Freese, 1959). *In vivo*, such analogs may be produced from normal cellular metabolism or by the action of chemical and physical factors, such as alkylating agents or ionizing radiation. In addition, synthetic base analogs with mutagenic or inhibitory properties have been used as tools for study of fundamental cellular processes, such as the metabolism of nucleic acid precursors or the mechanisms of DNA replication and repair. An important group of toxic and mutagenic analogs are the *N*-hydroxylated derivatives of purines and pyrimidines, such as 6-*N*-hydroxylaminopurine (HAP), 2-amino-HAP (AHAP) and *N*⁴-hydroxycytidine (see Khromov-Borisov, 1997). In particular, HAP has been shown to possess highly potent mutagenic effects in bacteria, yeast, and mammalian cells (Pavlov *et al.*, 1991; Kozmin *et al.*, 1998a; Barrett, 1981). HAP is believed to be incorporated like adenine or hypoxanthine via the purine salvage pathways and to be converted to a deoxynucleoside triphosphate (dHAPTP), serving as a precursor for enzymatic DNA synthesis, and inducing mutations due to its ambivalent coding capacity (Kozmin *et al.*, 1998a).

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Studies of the genetic control of HAP-induced mutagenesis in *Saccharomyces cerevisiae* and *Escherichia coli* has led to identification of HAP-hypersensitive mutants. In yeast, drastically enhanced sensitivity to the mutagenic and toxic effects of HAP was observed in *ham1* mutants (Pavlov, 1986; Noskov *et al.*, 1996). Ham1p has been shown to be a specific pyrophosphatase that acts to hydrolyze dHAPTP to the corresponding mononucleotide, hence preempting incorporation into the DNA (Kozmin *et al.*, 1998a; Hwang *et al.*, 1999; Burgis and Cunningham, 2007). While hydrolysis of dHAPTP is a major HAP-protective mechanism in yeast, it does not play a significant role in bacteria because inactivation of RdgB, the *E. coli* Ham1p homolog, does not confer HAP-sensitivity (Burgis *et al.*, 2003).

In *E. coli*, HAP-hypersensitivity was observed in strains carrying a deletion of the *uvrB-bio* chromosomal region (Pavlov *et al.*, 1996). The $\Delta(\textit{uvrB-bio})$ strains were also AHAP-hypermutable and sensitive to growth inhibition or killing by hydroxylamine (Pavlov *et al.*, 1996; Kozmin *et al.*, 1998b). Likewise, *Salmonella* strains carrying a corresponding deletion of the *uvrB-bio* region proved hypermutable by AHAP and N^4 -hydroxycytidine (Janion, 1978; Janion, 1979; Janion and Myszkowska, 1981). Our laboratory established that the base-analog hypersensitivity of *E. coli* $\Delta(\textit{uvrB-bio})$ mutants is due to a defect in biosynthesis of the Molybdenum Cofactor (MoCo), an essential cofactor for a variety of oxidoreductases (Kozmin *et al.*, 2000). Based on this result, we proposed that *E. coli* contains an as yet unidentified enzymatic activity that requires MoCo and is capable of inactivating HAP, presumably by some oxidation/reduction reaction.

MoCo is a pterin derivative, called molybdopterin (MPT), carrying a unique dithiolene group that coordinates a molybdenum atom acting as a catalytic redox center (for review, see Rajagopalan, 1996; Mendel and Bittner, 2006). At least four families of molybdoenzymes have been recognized based on sequence homology and the chemical structure of the Mo-containing redox center (Kisker *et al.*, 1999; Hille, 2002). The predominant family in *E. coli* and most other prokaryotes (the DMSO reductase family) utilizes a guanylated version of MoCo, named molybdopterin guanine dinucleotide (MGD). In contrast, enzymes of the xanthine oxidase and sulfite oxidase families, common to eukaryotes, utilize the guanylate-free MPT form of MoCo. The xanthine oxidase family is further characterized by the need for a MoCo maturation step, in which the molybdenum atom is additionally coordinated to an extra sulfur atom (MoCo sulfuration) (Mendel and Bittner, 2006). Interestingly, we recently showed that the base-analog protective mechanism in *E. coli* does not depend on MGD; instead, the MPT form of MoCo is sufficient (Kozmin and Schaaper, 2007). We also showed that deletion of all the known and putative molybdoenzymes of *E. coli* did not lead to base-analog sensitivity (Kozmin and Schaaper, 2007). Thus, it was proposed that perhaps an additional, as yet unrecognized, family of MoCo-dependent activities might exist in *E. coli* that is responsible for base-analog detoxification.

In the present work, we describe the isolation and characterization of novel HAP-sensitive mutants carrying defects in the *ycbX* and *yiiM* open reading frames. Genetic analysis of these mutants indicates that they control different sub-pathways within the overall MoCo-dependent pathway. The corresponding YcbX and YiiM proteins are characterized by a so-called MOSC domain, which has been found in eukaryotic MoCo sulfurases (Anantharaman and Aravind, 2002) and which may represent a novel MoCo-binding domain (Havemeyer *et al.*, 2006). From these and other data we propose that YcbX and YiiM represent novel MPT-containing molybdoenzymes in *E. coli*. Using *E. coli* cell-free extracts, we show that the main *ycbX/yiiM*-dependent HAP-inactivation pathways proceed by reduction to adenine.

Results

YcbX, a novel HAP-resistance determinant

As part of our efforts to better understand the mechanisms for the MoCo-dependent resistance of *E. coli* to *N*-hydroxylated base analogs, we searched for additional HAP-sensitive mutants beyond those affected in MoCo biosynthesis such as *moaE* or *moeA* mutants (Kozmin *et al.*, 2000; Kozmin and Schaaper, 2007). Several genome-wide searches using random transposon mutagenesis of the *E. coli* chromosome (see Experimental procedures) were performed, which yielded a number of HAP-sensitive mutants carrying a transposon insert in the *ycbX* open-reading frame. Some tests demonstrating the sensitivity of these mutants are shown in Figs. 1 and 2. In simple spot-tests (Fig. 1), a small aliquot of chemical is placed in the center of a plate containing the strain of interest and the plates are inspected for a zone of inhibition (or killing) after overnight growth. If desired, these plates can then be replicated on rifampicin-containing plates to obtain an estimate of the chemical-induced frequency of rifampicin-resistant mutants. In liquid incubation tests, growing cells are exposed to various concentrations of the chemical and the fraction of surviving cells is determined as a function of dose (Fig. 2). As seen in both Fig. 1 and 2, the *ycbX* defect leads to strongly increased sensitivity to both the toxic and mutagenic effects of HAP. On the other hand, the sensitivity of the new isolates was not as severe as that of the *moaE* or *moeA* mutants defective in MoCo biosynthesis. For example, in Fig. 1, the diameter of the inhibition zone is 50 mm and 35 mm in the *moaE* and *ycbX* strains, respectively. We also noticed certain specificity differences between the *ycbX* and MoCo defects. The *ycbX* mutant did not show any clear sensitivity to two other hydroxylated compounds, 2-amino-HAP (AHAP) and hydroxylamine (HA) to which MoCo-deficient strains are clearly sensitized (Figs. 3 and 4).

To investigate the genetic relationship between the *ycbX* and MoCo mutants we tested *ycbX moaE* double mutants. The result of the quantitative liquid incubation test showed that the sensitivity of the *ycbX moaE* double mutant was essentially indistinguishable from that of the single *moaE* mutant (Fig. 5). The *ycbX moaE* double mutant also proved as sensitive as the single *moaE* mutant when tested for AHAP and hydroxylamine using direct spot tests (data not shown). Consequently, it appears that *ycbX* likely controls a subpathway within the MoCo-dependent pathway, with a specificity that is geared towards HAP.

YcbX protein contains three essential conserved domains: β -barrel, MOSC and ferredoxin

Analysis of YcbX protein using the NCBI Conserved Domain Database (CDD) (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=cdd>) (Marchler-Bauer *et al.*, 2005) indicates the presence of three conserved domains: an N-terminal β -barrel domain, a central MOSC domain, and a C-terminal [2Fe-2S]-ferredoxin-like domain (Fig. 6). The first two domains, β -barrel and MOSC, were first found in eukaryotic MoCo sulfurases (the abbreviation MOSC was introduced by Anantharaman and Aravind [2002] and refers to MoCo sulfurase C-terminal domain). MoCo sulfurases transfer an inorganic sulfur from cysteine to the molybdenum center of MoCo as part of a final MoCo maturation (sulfuration) step. Such a sulfurated version of MoCo is utilized specifically by enzymes of the xanthine oxidase family, which includes xanthine dehydrogenase and aldehyde oxidase (Kisker *et al.*, 1999; Hille, 2002; Mendel and Bittner, 2006). Additional MOSC-containing proteins are distributed widely in pro- and eukaryotes; however, except for the MoCo sulfurases, all other members of the 'MOSC superfamily' are proteins without any confirmed function (Anantharaman and Aravind, 2002). Finally, the third domain of YcbX, ferredoxin, belongs to a widely distributed class of small iron-sulfur proteins serving as an electron carriers in various cellular processes (Sticht and Rösch, 1998). To check if all three YcbX domains are essential for YcbX function, we constructed a set of strains carrying in-frame deletions of each of the domains (see Fig. 6 and Experimental procedures). When testing for HAP-sensitivity we found that deletion of any

YcbX domain led to HAP-hypersensitivity, in a manner indistinguishable from a complete *ycbX* deletion ($\Delta ycbX$) in strain NR16262 (Fig. 7).

YiiM, a second *E. coli* MOSC-containing protein, is also involved in protection against N-hydroxylated analogs

Homology searches have predicted the existence of a second MOSC-containing protein in *E. coli*, YiiM (Anantharaman and Aravind, 2002). YiiM is a predicted 224-amino acid protein composed of a MOSC domain and a C-terminal domain with an assumed trihelical structure (Anantharaman and Aravind, 2002). To check for its possible role in base-analog detoxification we created a *yiiM* deletion strain (see Experimental procedures) and found that it displayed moderately increased sensitivity to HAP (Fig. 1A) as well as significantly enhanced AHAP-induced mutagenesis (Fig. 3B). Moreover, the *yiiM* mutant, in contrast to the *ycbX* mutant, was sensitized to hydroxylamine, resembling, in fact, the MoCo-deficient strain (Fig. 4). A double *ycbX yiiM* mutant showed a significant increase in HAP- and AHAP-sensitivity relative to each of the single mutants (Figs. 1 and 3). This observation, as well as the noted differences between *ycbX* and *yiiM* with respect to HAP, AHAP, and hydroxylamine (compare Figs. 1, 3, and 4), leads us to propose that the two genes likely participate in two different pathways within the base-analog protective mechanisms (see Fig. 9).

Interestingly, we noted that the combination of *yiiM* and *moaE* increased HAP-sensitivity relative to the single *moaE* strain (Fig. 5), suggesting that *yiiM* may also work outside the MoCo pathway; moreover, a triple *ycbX yiiM moaE* mutant was more HAP-sensitive than the *yiiM moaE* strain (Fig. 5), suggesting that at least in the *yiiM moaE* background *ycbX* may also have an additional function. These results suggest that while *ycbX*, and presumably also *yiiM*, are clearly involved in the MoCo-dependent pathway, both genes also may function to some extent in a MoCo-independent manner.

YcbX and YiiM are not involved in MoCo biosynthesis

The possibility needed to be considered that the *ycbX* and *yiiM* genes are involved indirectly in base-analog detoxification through involvement in an as yet unrecognized aspect of MoCo biosynthesis, an area still subject of continuing investigation (see Mendel and Bittner, 2006). A common phenotype of *E. coli* mutants defective in the MoCo biosynthesis is resistance to chlorate under anaerobic conditions (Shanmugam *et al.*, 1992). In wild-type strains, but not MoCo-deficient strains, chlorate is converted to toxic chlorite due to the action of several molybdoenzymes of the DMSO reductase family, such as nitrate reductase and trimethylamine *N*-oxide (TMAO) reductase (Stewart and MacGregor, 1982). We found that the *ycbX* (NR15873), *yiiM* (NR15871), and double *ycbX yiiM* (NR15876) strains were fully sensitive to chlorate similar to a wild-type strain (NR10836), even at a very low chlorate concentration, 0.5 mM (data not shown). We also tested for the activity of nitrate and TMAO reductases using a simple colony pigmentation test using MacConkey (MC) indicator plates supplemented with nitrate or TMAO (Stewart and MacGregor, 1982; and Experimental procedures). On these media, usage of the substrates (nitrate or TMAO) leads to acidification of the colonies and, hence, altered coloration of the colonies (Stewart and MacGregor, 1982). We observed that on MC plates containing nitrate or TMAO, the MoCo-deficient strain *moeA* formed deep-red colonies, whereas wild-type, *ycbX*, *yiiM*, and *ycbX yiiM* strains formed light salmon-colored colonies (data not shown). In addition, TMAO reduction in the wild-type, *ycbX*, *yiiM*, and *ycbX yiiM* strains was easily detectable by formation of the odorous product trimethylamine, which was not detectable in the MoCo-deficient mutant. Taken together, these experiments indicate that neither *ycbX* nor *yiiM* mutants have any significant defects in MoCo (MGD) biosynthesis.

A limited involvement of Biotin Sulfoxide Reductase in base-analog detoxification

As seen from Figs. 1 and 3, the double *ycbX yiiM* mutant was less sensitive to HAP and AHAP than a MoCo-deficient strain. This suggested that there might exist one or more additional MoCo-dependent pathways in addition to those controlled by *ycbX* and *yiiM*. In previous work (Kozmin and Schaaper, 2007) we demonstrated that the major base-analog protective mechanism utilizes the GMP-free molybdopterin (MPT) version of MoCo rather than the common molybdopterin-guanine-dinucleotide (MGD). This can be seen, for example, in Figs. 1 and 3 where the *moaA* strain (defective in conversion of MPT to MGD) is shown to be resistant to HAP and normally sensitive to AHAP. However, in the *ycbX yiiM* background, the *moaA* defect clearly sensitizes the strain such that it is now indistinguishable from the *ycbX yiiM moaE* mutant (Figs. 1,3,5). Thus, the additional pathway(s) are *moaA*-dependent and are predicted to utilize the MGD version of MoCo. To find this responsible MGD-utilizing activity, we systematically combined the *ycbX yiiM* double deficiency with deletions of the genes encoding the 13 known and putative MGD-requiring enzymes (strains NR16047 through NR16060) (see Table1, and Kozmin and Schaaper, 2007). This experiment showed that only the combination of *yiiM ycbX* with *bisC* (encoding Biotin Sulfoxide Reductase) (del Campillo-Campbell and Campbell, 1982; Pierson and Campbell, 1990) increased HAP- and AHAP-sensitivity. Moreover, the *yiiM ycbX bisC* strain is indistinguishable from the *ycbX yiiM moaA* or *ycbX yiiM moaE* strains (see Figs. 1,3,5), indicating that no additional factors are required to account for the sensitivity of the MoCo mutants. Note that no contribution of the *bisC* pathway to HAP resistance was detected in a MoCo-proficient background (Kozmin and Schaaper, 2007). Therefore, the *bisC* pathway must be considered a minor pathway. The overall scheme of HAP detoxification based on our results is presented in Fig. 9

Base-analog sensitivity of *ycbX* and *yiiM* strains is not due to lack of MoCo sulfuration

While *ycbX* and *yiiM* are not required for MoCo biosynthesis, it cannot be excluded that the genes are involved in some form of MoCo modification specific for the base-analog detoxification. For example, since *ycbX* and *yiiM* share the MOSC domain with the eukaryotic MoCo sulfurases involved in maturation (sulfuration) of MoCo used by members of the xanthine oxidase family (Kisker *et al.*, 1999; Hille, 2002), the possibility of a role of YcbX and YiiM in MoCo sulfuration in *E. coli* was considered. To test this hypothesis, we developed an *in vivo* assay for the activity of human xanthine dehydrogenase (hXDH) expressed in *E. coli*. A cDNA clone of hXDH was prepared as described in Experimental procedures. The test for activity was based on the inability of *E. coli guaB* (guanine or xanthine) auxotrophs to utilize hypoxanthine as a GMP source (see Fig. 8A), consistent with the reported absence of physiologically significant XDH activity in *E. coli* (Xi *et al.*, 2000). However, conversion of hypoxanthine to xanthine by hXDH may overcome this deficiency. The results in Fig. 8B show that expression of hXDH indeed permitted growth of the *guaB* strain on hypoxanthine (purple color indicative of active respiration; see Legend for details). Allopurinol, a specific inhibitor of xanthine dehydrogenase (Elion, 1989) prevented the use of hypoxanthine (data not shown). In the MoCo-deficient *moaE* strain no hypoxanthine utilization was detected, confirming the dependence of XDH on MoCo (Fig. 8B). Importantly, neither the single *ycbX* or *yiiM* deficiencies nor the combined *ycbX yiiM* deficiency inhibited this activity (Fig. 8B). In view of the well-documented dependence of xanthine dehydrogenase on MoCo sulfuration (see Mendel and Bittner, 2006), we conclude that, while sulfuration appears to occur in *E. coli*, the *ycbX* and *yiiM* genes do not play a significant role in this maturation process.

HAP-detoxification by conversion to adenine

As part of our efforts to elucidate the HAP-detoxification pathways, we further addressed the reaction by which HAP might be converted to less harmful compounds. One plausible reaction for this conversion is reduction to adenine. Reduction of HAP to adenine was previously

demonstrated *in vitro* by mammalian xanthine oxidase (Clement and Kunze, 1992). While our experiments show that xanthine dehydrogenase is not involved in this reaction in *E. coli*, it is reasonable to assume that other MoCo-dependent activities could perform this reaction as well. Secondly, studies in our laboratory with *E. coli* purine auxotrophs (*pur* strains) have indicated that these strains can use HAP as a purine source in a manner suggestive of prior conversion to adenine (data not shown). We tested this hypothesis by following the fate of HAP (and adenine) in *E. coli* cell free extracts (see Experimental procedures). Initial experiments indicated in these extracts that both HAP and adenine were efficiently deaminated to yield hypoxanthine. We therefore constructed strains lacking the genes for both adenosine deaminase (*add*) and (cryptic) adenine deaminase (*ade*, previously called *yicP*) (Zalkin and Nygaard, 1996; Matsui *et al.*, 2001). The outcome of the reactions was monitored by thin-layer chromatography (TLC) or UV spectroscopy (exploiting the differential λ_{max} values for HAP, adenine, and hypoxanthine, 268, 260, and 250 nm, respectively) (See experimental procedures). An example of the UV experiments is presented in Fig. 10. The $\Delta\text{add} \Delta\text{ade}$ cell extracts are capable of converting 1 mM HAP completely to adenine within 40 min of incubation at 30° C (Fig. 10B, E). Importantly, under the same conditions, no HAP conversion was observed in the corresponding *moa* or *ycbX yiiM* cell extracts (Fig. 10C, D, E), while the single *ycbX* and *yiiM* extracts provided reduced activities (Fig. 10E). Thin-layer chromatography results fully confirmed these findings (not shown). Thus, these results (i) support the contention that the major detoxification reaction for HAP is reduction to adenine, and (ii) that this reaction requires participation of MoCo, YcbX, and YiiM, as suggested by our genetic experiments.

Discussion

N-hydroxylated derivatives of the normal purine or pyrimidine bases, such as 6-*N*-hydroxyaminopurine (HAP) or *N*⁴-hydroxycytidine, are potent mutagens in organisms from bacteria to man. At the same time organisms possess, at least as revealed in yeast and bacteria, efficient mechanisms to prevent the toxic and mutagenic effects of these agents. In *E. coli*, this protective system requires the molybdenum cofactor (MoCo). The system is remarkably effective as can be estimated based on observed survival and mutagenesis data: when growing overnight in minimal media, *moa* or *moe* strains are effectively killed by concentrations of around 0.01 $\mu\text{g/ml}$ HAP, while a wild-type strain readily sustains growth in media containing up to 100 $\mu\text{g/ml}$ (data not shown). In the same experiments, high levels of mutations are obtained for MoCo-deficient strains at 0.001 $\mu\text{g/ml}$ of HAP, while wild-type strains require at least 1 $\mu\text{g/ml}$ for similar mutagenesis levels. Thus, the MoCo-dependent system is capable of reducing the sensitivity of *E. coli* by 1,000- to 10,000-fold. Our present study establishes a genetic framework delineating the factors involved in this detoxification mechanism and, secondly, provides evidence that the newly discovered YcbX/YiiM pathways operate through an enzymatic reduction of HAP to adenine.

The genetic framework as outlined in Fig. 9 emphasizes as particular feature that the two main MoCo-dependent pathways are dependent on the MPT form of MoCo rather than the common MGD form found in *E. coli* and related bacteria. This dependence on MPT, combined with our observation that none of the known and putative molybdoenzymes of *E. coli* significantly affect base-analog resistance (Kozmin and Schaaper 2007), suggests that resistance is mediated by a novel group of smolybdoenzymes. In the simplest case, the YcbX and YiiM proteins themselves represent these novel molybdoactivities. The two proteins are characterized by possession of a MOSC domain (Amrani *et al.*, 2000; Anantharaman and Aravind, 2002) first recognized in eukaryotic MoCo sulfurases. While the MOSC domain appears to be essential for *in vivo* MoCo sulfuration (Dai *et al.*, 2005; Sagi *et al.*, 2002; Peretz *et al.*, 2007), its precise function is not known. Both a sulfur-carrier and a MoCo hosting function have been considered (Amrani *et al.*, 2000; Anantharaman and Aravind, 2002). Assuming that YcbX and YiiM

represent the novel molybdoenzymes, the MOSC domain is a likely site for hosting MoCo and acting as catalytic center.

The above model is fully consistent with important recent findings by Havemeyer *et al.* (2006) in a mitochondrial system. These investigators studied a mitochondrial prodrug converting system and discovered a novel activity, operating in conjunction with cytochrome b₅ and NADH:cytochrome b₅ reductase, capable of converting the prodrug benzamidoxime to its active form benzamidine. This conversion is analogous to the conversion of HAP to adenine, as it entails the reduction of an *N*-hydroxyl compound (benzamidoxime) to the corresponding amino form (benzamidine). Most interestingly, the newly isolated 35 kDa protein was shown to possess a MOSC domain and to be able to bind MoCo. Thus, it appears that a hitherto undescribed but apparently broadly-distributed class of molybdoenzymes exists, as judged from their discovery in both bacteria and mammalian cells. The precise function(s) of these enzymes remain to be determined, and may well extend beyond the examples provided by these current cases.

The scheme of Fig. 9 also shows the detoxification step mediated by the MGD-requiring enzyme biotin sulfoxide reductase (BisC). The function described so far for *E. coli* BisC is the reduction of biotin sulfoxide (del Campillo-Campbell and Campbell, 1982; Pierson and Campbell, 1990) or methionine sulfoxide (Ezraty *et al.*, 2005). Our present result suggests that its specificity may be broader, as also reported for the biotin sulfoxide reductase from *Rhodobacter* (Pollock and Barber, 1997). Nevertheless, the action of BisC in the HAP detoxification is only minor, as its effect was only discerned in the *ycbX yiiM* background and, also, its activity was not observed in the *in vitro* experiments of Fig. 10.

Of interest with regard to the scheme of Fig. 9 are the different specificities that appear associated with each of the subpathways. While *moa* or *moe* strains display increased sensitivity to all three agents (HAP, AHAP, and hydroxylamine), the *ycbX* pathway is most effective towards HAP, while the *yiiM* pathway is most effective for AHAP and hydroxylamine (Figs. 1, 3, 4). Hydroxylamine, while not a base analog, represents the simplest *N*-hydroxylated compound and may be a useful model compound for further studies. Reduction of hydroxylamine in *E. coli* has been reported mediated by the hybrid cluster protein (Wolfe *et al.*, 2002) or by cytoplasmic cytochrome c nitrite reductase (Angove *et al.*, 2002), neither of which are molybdoenzymes. Thus, a novel pathway for dissimilation of hydroxylamine has been discovered. The specificity differences of Fig. 9 may reflect differences in substrate preference for, say, YcbX vs. YiiM protein, but may also reflect different stages in nucleotide metabolism at which the proteins may act. This will be an important area for further studies.

As noted in the Results, our data leave open the possibility that the *ycbX* and *yiiM* genes play additional roles in base-analog detoxification outside the MoCo-dependent pathways. For example, in Fig. 5, the *moaE yiiM* strain was more HAP-sensitive than the single *moaE* mutant, while the triple *moaE yiiM ycbX* was more sensitive than the double *moaE yiiM*, suggesting that both *ycbX* and *yiiM* might have additional function(s). On the other hand, the *moaE ycbX* double was as sensitive as the single *moaE* mutant, arguing against an additional function for *ycbX*. These issues will require further study, and both direct and indirect effects may be operative. However, from the present perspective the most important finding is that the triple *ycbX yiiM mobA* or *ycbX yiiM bisC* mutants are as sensitive as the *ycbX yiiM moaE* strain (as seen in Figs. 1, 3, and 5). This result indicates that after inactivation of BisC (via *bisC* or *mobA*) the *yiiM* and *ycbX* deficiencies completely eliminate the *moa*-dependent base-analog resistance.

The conversion of HAP to adenine as demonstrated in cell-free extracts (Fig. 10) is consistent with other genetic data from our laboratory on the usage of HAP as a purine source by *E.*

coli purine auxotrophs (data to be published elsewhere). In brief, *pur add* strains defective in both *de novo* purine biosynthesis (*pur*) and adenosine deaminase (*add*) are capable of using adenine as a purine source based on conversion of adenine to AMP by Apt activity (adenine phosphoribosyl transferase) (Zalkin and Nygaard, 1996). This strain can also use HAP as purine source, and this usage, like that of adenine, is blocked by an additional *apt* defect. The simplest interpretation is that, in this kind of experiment, HAP is first converted to adenine by the YcbX/YiiM system prior to its usage in purine salvage. Note that it is not possible to demonstrate the absence of this type of HAP usage in MoCo (or *ycbX/yiiM*) deficient cells, because these cells are effectively killed by the purine concentrations necessary for bacterial growth (10-50 µg/ml). An interesting parallel to this reductive usage of HAP are the early studies by Popowska and Janion (1975,1977). Using toluenized *E. coli* cells these investigators concluded that the base analog *N*⁴-hydroxycytidine could be used as pyrimidine source via reduction to cytidine by an unknown system. Thus, it appears that an *N*-reductive reaction is a current hallmark of the activity of the MOSC systems in both bacteria and higher organisms.

It is an interesting question why *E. coli* would possess a defensive system against *N*-hydroxylated base analogs. It has been suggested that HAP can be produced occasionally as a byproduct of normal cellular metabolism (Lieberman, 1956; Budowski, 1976; Clement and Kunze, 1990). If so, its efficient removal from the cell would be desirable. On the other hand, MoCo-deficient strains are not spontaneous mutators, at least under the conditions that we have tested. It is possible that increased amounts of *N*-hydroxylated compounds will be formed under more stressful situations, such as oxidative stress conditions; a mutator effect of MoCo-deficient strains might be observed under those conditions. We note that exposure of DNA and nucleobases to peroxy radicals was reported to yield HAP as the major product (Simandan *et al.*, 1998). Alternatively, as the MoCo-dependent system converts the hydroxylated bases to the normal bases, the system might primarily have a salvage or scavenging function. Also, as inferred perhaps from the mitochondrial data of Havemeyer *et al.* (2006), the bacterial system may be geared towards a broader range of *N*-hydroxylated compounds. *N*-hydroxylated compounds are intermediates in both the oxidation of aromatic amines (by cytochrome P450s) and the reduction of aromatic nitro compounds (by nitroreductases) These *N*-hydroxylated derivatives are unstable and highly reactive and can produce DNA adducts (Rosenkranz and Mermelstein, 1983).

The present study and that of Havemeyer *et al.* (2006) suggest that MOSC-containing proteins represent a novel class of molybdoenzymes with similar biochemical properties in bacteria and mammals. The precise biological function of these molybdoenzymes is not yet known. However, an indispensable role of these proteins in base-analog detoxification in bacteria is clear. It is possible that these molybdoenzymes play a role in protection against mutagenic base analogs and related carcinogens in humans as well, providing an incentive for further research in this area.

Experimental procedures

Bacterial strains and constructions

The *E. coli* strains used in this study are listed in Table 1 along with their source or derivation. P1 transductions were performed using P1*virA*. All mutagenesis and base-analog sensitivity tests were performed using strain NR10836 (Kozmin *et al.*, 2000) and its mutant derivatives. The *ycbX::mini-Tn10cam* allele was obtained initially in strain KA796 upon random transposon insertion mutagenesis, as described in a section below, and then transferred to NR10836 by P1 transduction, yielding strain NR15873. Construction of the *ycbX* in-frame deletions (NR16258, NR16259, NR16261, and NR16262) is described in a separate section below. A deletion of *yiiM* was initially generated in strain BW25113 (Datsenko and Wanner, 2000) by the PCR-based gene replacement method, using the Kan^r module of plasmid pKD13

as a template (Datsenko and Wanner, 2000). Primers for the PCR were: *yiiM*-p1: 5'-ttg atg tat aca cag gca aga ttc agg ctt atc ccg aag gca aac cca gcg tgt agg ctg gag ctg ctt c-3' and *yiiM*-p4: 5'-taa ccc ggc agc gga gag taa acg gtg ata ttg atc gtc atc aaa cgg cac tgt caa aca tga gaa tta a-3'. The resulting $\Delta yiiM::kan$ deletion-insertion was transferred into strain NR10836 by P1 transduction using P1*virA*, yielding strain NR15870. To obtain strain NR15871 ($\Delta yiiM$), the Kan^r insert in *yiiM* was eliminated by induction of recombination between repeats flanking the *kan* gene, using plasmid pCP20 (Datsenko and Wanner, 2000). NR10836 derivatives carrying deletions of known and putative molybdoenzymes ($\Delta bisC::kan$, $\Delta dmsA::kan$, $\Delta torA::tet$, $\Delta torZ::kan$, $\Delta narG::tet$, $\Delta narZ::kan$, $\Delta napA::kan$, $\Delta fdnG::kan$, $\Delta fdhF::kan$, $\Delta fdoG::tet$, $\Delta ynfE::kan$, $\Delta ynfF::kan$, $\Delta ydeP::kan$) have been described (Kozmin and Schaaper, 2007). These deletions were transferred into strain NR15876 (*ycbX::mini-Tn10cam*, $\Delta yiiM$) by P1 transduction yielding strains NR16047 through NR16060 (Table 1). Activity of the human xanthine dehydrogenase (see below) was tested in a modified version of strain BL21(DE3) (Table 1) constructed as follows. First, we noted that strain BL21(DE3) (Studier and Moffat, 1986) is chlorate-resistant and HAP-sensitive, suggesting a defect in MoCo biosynthesis (Kozmin *et al.*, 2000). On the assumption that this phenotype might be associated with the *gal* defect in BL21 (noting that *gal* is located very close to the *modABC* and *modEF* operons (Berlyn, 1998), which are important for molybdenum transport and cofactor biosynthesis) (Shanmugam *et al.*, 1992; Kozmin *et al.*, 2000), we created a *gal*⁺ derivative of BL21 by P1 transduction. The *gal*⁺ derivative NR15967 proved chlorate-sensitive and HAP-resistant, consistent with our hypothesis. NR15967 was then lysogenized with bacteriophage λ DE3 using the lysogenization kit from Novagen, followed by introduction of the $\Delta mobA::kan$ allele from strain NR15994 and elimination of the *kan* insert using plasmid pCP20 (Datsenko and Wanner, 2000). Finally, to yield the strain NR17070, we introduced the $\Delta guaB::tet$ allele by P1 transduction. The $\Delta guaB::tet$ allele was created in strain BW25113 by the PCR-based gene replacement method of Datsenko and Wanner (2000) using primers *guaB*-p1T 5'-aaa cag ata ata taa atc gcc cga cat gaa gtc ggg cga aga gaa tca ggA AGA GGG TCA TTA TAT TTC G-3' and *guaB*-p4T 5'-taa tgc cgc ggc aat att tat taa cca ctc tgg tgc aga tat tgc cca tgA CTC GAC ATC TTG GTT ACC G-3' (upper case letters indicate the sequences used to amplify the 2-kb Tet^r module containing the *tetR* and *tetA* genes from transposon Tn10). The $\Delta add::kan$ and $\Delta yicP::cam$ ($\Delta ade::cam$) deletion/insertions were created in strain BW25113 by the method of Datsenko and Wanner (2000) using primers *add*-p1 5'-ttg atg gca aca ttc gtc ccc aga cca ttc ttg aac ttg gcc gcc agt atg tgt agg ctg gag ctg ctt c-3' plus *add*-p4 5'-gtt aat gct ggc acg aat gcc atg ctc aag gaa cgt ttt cag cgg atg tgc tgt caa aca tga gaa tta a-3' (using pKD13 as a template), and *yicP*-Cm1 5'-ttc ttt atg ata aat aat aat caa att gat aaa atc aaa atg aga aaa atgtg tag gct gga gct gct tgc-3' plus *yicP*-Cm2 5'-tgg cat aaa cgt aac tgg tga ctt ttg ccc ggc atg acg cgg ggc ttt ttc atg aat atc ctc ctt ag-3' (using pKD3 as a template), respectively. The $\Delta add::kan$ and $\Delta yicP::cam$ alleles were then transferred into the NR10836 background by P1 transduction (see Table 1).

Media and chemicals

Bacteria were cultivated in LB broth (Miller, 1972) or minimal Vogel-Bonner medium (VB) (Vogel and Bonner, 1956) containing 0.2% glucose as carbon source. Solid media contained 1.5% agar. For selection of antibiotic-resistant clones, LB medium was used containing 35 μ g/ml of kanamycin, 20 μ g/ml of chloramphenicol, 15 μ g/ml of tetracycline, or 100 μ g/ml of rifampicin. MacConkey (MC) indicator plates for monitoring nitrate reduction or trimethylamine *N*-oxide reduction were prepared according to Stewart and MacGregor (1982). HAP was purchased from ICN Biochemicals; 2-amino-HAP was synthesized from 2-amino-6-chloropurine according to the method of Giner-Sorolla and Bendich (1958) by Ilya Kuchuk, Indiana University. HAP and AHAP were used as the free base. Hydroxylamine hydrochloride and other chemicals were purchased from Sigma-Aldrich.

Isolation and identification of HAP-hypersensitive mutants by random transposon insertion mutagenesis

A library of random transposon insertions was obtained in strain KA796 using the mini-Tn10cam transposon from phage λNK1324 as described by Kleckner *et al.* (1991). Chloramphenicol-resistant clones were restreaked on minimal VB plates containing 0 or 10 μg/ml of HAP to search for HAP-sensitive mutants. Genomic DNA from HAP-sensitive clones was isolated using Easy-DNA kit (Invitrogen). To determine the location of transposon insertions we used arbitrary primed PCR (Caetano-Anolles, 1993). Briefly, a first round of PCR was performed on genomic DNA of each mutant using three primers: two primers with random 3' ends, ARB1 (5'-ggc cac gcg tcg act agt acN NNN NNN NNN gat at-3') and ARB6 (5'-ggc cac gcg tcg act agt acN NNN NNN NNN acg cc-3'), and a third primer complementary to the mini-Tn10cam sequence, CmExt (5'-cag gct ctc ccc gtg gag g-3'). A second round of PCR was then performed with primers ARB2 (5'-ggc cac gcg tcg act agt ac-3') and CmInt (5'-ctg cct ccc aga gcc tg-3'). The final PCR product was purified with a QIAquick spin PCR purification kit (Qiagen) and sequenced using ARB2 primer and the dRhodamine terminator cycle DNA sequencing kit (PE Biosystems, Warrington, UK).

Construction of the ycbX in-frame deletion alleles

YcbX protein contains three predicted domain, β-barrel, MOSC, and ferredoxin (see Text). To create individual in-frame deletions of each of these domains, we used the PCR-based gene replacement method of Datsenko and Wanner (2000). First, we generated kanamycin-resistant deletion-insertions for each domain in strain BW25113. These kanamycin-resistant alleles were transferred into strain NR10836 by P1 transduction. The *kan* modules were then eliminated by induction of recombination between the repeats flanking the *kan* gene using plasmid pCP20 (Datsenko and Wanner, 2000). The resulting in-frame deletions were verified by DNA sequencing. As shown in Fig. 6, after elimination of the Kan^r module, the in-frame deletion alleles encode reduced versions of the YcbX protein containing small (27- to 30-residue) polypeptide in place of the deleted domain (see Legend to Fig. 6). To generate the initial deletion-insertion alleles, plasmid pKD13 was used as a template (Datsenko and Wanner, 2000) and the following primer pairs (upper case letters indicate pKD13 sequence): for Δ*ycbX* [Δβ-barrel]: YcbX-ΔN-p1 (5'-gcg ttt cac gcg ccg ggt cat ttg tgg ccc cac cca gcg taa ttg cac tt GTG TAG GCT GGA GCT GCT TCG-3') and YcbX-ΔN-p4 (5'-cgt cat gtc ata aag ttg agg gct tat ttt cat ttg agg acc gca ccg tg ATT CCG GGG ATC CGT CGA CC-3'); for Δ*ycbX*[ΔMOSC]: YcbX-ΔMOSC-p1 (5'-atc atc agc ggc agc tgc gcc gta aat ttt tgc cgg agc cgt tgc cag aa GTG TAG GCT GGA GCT GCT TCG-3') and YcbX-MOSC-p4 (5'-ccc gcg aag tgc aat tac gct ggg tgg ggc cac aaa tga ccc ggc gcg tg ATT CCG GGG ATC CGT CGA CC-3'); and for Δ*ycbX* [ΔFerredoxin]: YcbX-ΔFer-p1 (5'-gaa acc gca ggt taa tgt tga cag ctt cag cct cga aca ggc agt cta ac GTG TAG GCT GGA GCT GCT TCG-3') and YcbX-Fer-p4 (5'-ctg ccg ctg atg ata ccg cca aca tca cgc aac aac cgg acg caa atg ta ATT CCG GGG ATC CGT CGA CC-3'). To obtain a complete deletion of *ycbX* (denoted as Δ*ycbX* in Table 1), we used primers YcbX-ΔN-p1 and YcbX-Fer-p4. After elimination of the Kan^r modules using plasmid pCP20 (Datsenko and Wanner, 2000), the deletion alleles were confirmed by DNA sequencing via PCR amplification using primers *ycbX*-seqA (5'-aac cct gcg ttg tta ttg cg-3') and *ycbX*-seqB (5'-tga gga ttt cga gca taa cc-3'). Sequencing was done both directions using the same primers, *ycbX*-seqA and *ycbX*-seqB.

Spot-tests for base-analog sensitivity

Stationary *E. coli* cultures grown in LB were diluted 30-fold in 10 ml of 0.9% NaCl in Petri dishes and transferred to VB minimal plates using a multi-prong replicator device (approximately 0.1 ml total per plate). After the spots had dried, a few microliters of a solution of HAP or AHAP (50 μg in DMSO) or hydroxylamine (150 μg in water) were spotted onto the

center of the plate. The pH of the hydroxylamine-HCl solution was ~3.4. Identical results were obtained with either this solution or solutions neutralized to pH 7.0. The plates were incubated overnight at 37° C and inspected the next day for zones of inhibition. In some experiments, the plates were further replicated to LB plates containing rifampicin, incubated overnight at 37° C, and inspected for the induction of Rif^r colonies surrounding the place of mutagen placement.

Tests for growth inhibition by base analogs in liquid cultures

A single colony of each strain to be tested was inoculated into 5 ml of liquid LB and grown overnight at 37° C. 80 µl of each culture was inoculated into 40 ml of minimal VB medium (supplemented with 1 µg/ml of thiamine) in 125-ml flasks, and incubated for 6-7 hrs at 37° C at 200 rpm with shaking to reach mid-log-phase (OD₆₀₀ = 0.2, ~ 10⁸ cells/ml). At this point, for each strain and each concentration of HAP to be tested, three 1-ml aliquots were transferred into 13×100 mm glass culture tubes. An appropriate amount of HAP water solution (0.1 mg/ml) was added to each tube. Cultures were incubated for 2 hrs at 37° C on a rotating drum at 56 rpm and then placed on ice. The cultures were diluted 10⁴-fold in 0.9% NaCl and appropriate volumes of the dilution were plated on LB plates in triplicate to count survivors. Plates were incubated 20-24 h at 37°C. The fraction of surviving cells was determined by dividing the average number of cells in HAP-exposed cultures divided by the average number in the non-exposed cultures. For each strain, this test was repeated 2-3 times, and average survival values were used to obtain survival curves (Fig. 2).

Mutant frequency determinations

For each strain and each HAP concentration, 10 independent 1-ml LB cultures were started from 10⁴ cells. The cultures were grown overnight at 37° C with agitation. Mutant frequencies were determined by plating appropriate culture volumes on LB-rifampicin plates (to obtain the number of Rif^r cells per culture) and 100 µl of 10⁻⁶ dilution on LB plates (to obtain the total number of cells per culture).

Tests for chlorate resistance and nitrate or TMAO reduction

To test for chlorate resistance, approximately 10³ cells were plated on LB plates containing either 0.5 mM or 25 mM KClO₃. The plates were incubated overnight at 37° C under anaerobic conditions using a Becton Dickinson BBL gas pack anaerobic system, after which they were incubated aerobically for an additional 6-10 hrs. Under these conditions, chlorate-sensitive strains do not form colonies, whereas chlorate-resistant strains plate with essentially 100% efficiency. To monitor the ability of cells to reduce nitrate or trimethylamine-*N*-oxide (TMAO), we plated approximately 10² cells on MC indicator plates supplemented with either 0.1 M KNO₃ or 0.1 M TMAO. The plates were incubated aerobically overnight at 37° C and inspected for red pigmentation of the colonies indicative of nitrate reductase or TMAO reductase activities.

Cloning of the human xanthine dehydrogenase (hXDH)

'PCR Ready First Strand cDNA' (BioChain, cat# C1234226-10) from human normal adult small intestine was used as source of the hXDH-encoding cDNA. Amplification was performed in three consecutive rounds using *PfuTurbo*® DNA polymerase (Stratagene) using conditions recommended by the supplier. In round I, we amplified separately the left and right arms (2 kb each) of the hXDH-encoding cDNA using two primers pairs: hXDH-*NdeI* (5'-atc cta cca tat gac agc aga caa att gg-3') plus hXDH-int2 (5'-tat gcc caa cac aag taa cc-3'), and hXDH-*BglII* (5'-ata aag atc ttt aga ccc tca cag acc agg-3') plus hXDH-int1 (5'-atg atg aga cag tct ttg cg-3'). The int1 and int2 primers were chosen such as to generate an internal 3' overlap between the two arms. In round II, the entire amounts of purified PCR products from round I were mixed

together and further amplified in the presence of the two external primers, hXDH-*NdeI* and hXDH-*BglII*. Finally, to obtain sufficiently large amounts of the final full-length (4-kb) product, in round III 1/50 volume of reaction II was used as a template for amplification with primers hXDH-*NdeI* and hXDH-*BglII*. The resulting 4-kb PCR product corresponding to the hXDH-encoding DNA was gel-purified and restricted with *NdeI* and *BglII* restriction endonucleases. The resulting fragment was cloned into the *BamHI* and *NdeI* sites of the pET3a expression vector (Novagen) to yield plasmid pET3a-hXDH, in which the hXDH coding sequence is placed under control of the T7 promoter. The final construct was verified by DNA sequencing.

In vivo test for the hXDH activity using tetrazolium violet indicator

In this test we monitored the ability of *guaB* strains expressing hXDH to utilize hypoxanthine as a GMP source (see Fig. 8A) in the presence of the tetrazolium violet (TV) indicator dye. In growing actively respiring cultures TV (a colorless substance) is efficiently reduced to a deep-purple insoluble formazan derivative, whereas in starving cultures this reduction does not take place (Bochner and Savageau, 1977;Bochner *et al.*, 2001). Thus, strains NR17070 and NR17072 through NR17075 (Table 1) were transformed with plasmids pET3a (control) and pET3a-hXDH. A single colony of each transformant to be tested was inoculated into 6 ml of LB supplemented with 200 µg/ml of ampicillin, and incubated overnight at 37°C with agitation. Cells from these cultures were collected by centrifugation and resuspended in 20 ml of minimal VB media containing 100 µg/ml of ampicillin, 40 µg/ml of guanosine, 1 mM sodium molybdate, 0.02 mM IPTG, and incubated for 24 hrs at 16° C with agitation. After that, cells were washed and resuspended in 2 ml of indicator media (minimal VB medium containing 0.01% TV, 0.02 mM IPTG, and 40 µg/ml of either hypoxanthine or xanthine) at a density $OD_{600} = 0.1$, and incubated for 2-3 days at room temperature without shaking and inspected for purple pigment precipitation.

Conversion of HAP to adenine in cell-free extracts

Cell-free extracts were prepared according to Lu *et al.* (1983). 500-ml LB cultures were grown to $OD_{600} = 1.0-1.2$. Cells were collected by centrifugation, and each gram of cells was resuspended in 1.5 ml of 50 mM Tris-HCl (pH 7.6) containing 10% sucrose. Cell suspensions were quickly frozen in a dry ice/ethanol bath. For thawing, the frozen suspensions were placed at 0°C for 20 min and then in a 20° C waterbath until just melted. DTT, KCl, and lysozyme were added to final concentrations of 1.2 mM, 150 mM, and 0.23 mg/ml, respectively. incubation was continued for 1 h at 0° C followed by placement in a 37° C bath until the temperature of the solution reached 20° C. Cell debris was removed by centrifugation for 1 h at 37,000 rpm at 4° C. Supernatants (100 µl-aliquots) were stored at -80° C. Total protein concentration in the extracts was determined using the Non-Interfering Protein Assay Kit from Calbiochem. Extracts prepared by this procedure contained 6-9 mg of protein per ml. To assay for activity, 2 µl of a 100 mM HAP solution in DMSO or 2 µl of DMSO (control reaction) was added to 200 µl of cell-free extract, and the reaction mixtures were incubated at 30° C. Every ten minutes, 20-µl samples were removed and heat-inactivated for 2 min at 80° C. Samples were chilled on ice, and centrifuged for 5 minutes at 14,000 rpm in a microcentrifuge. 15-µl supernatant samples were removed and used for further analysis. The reaction products were monitored by UV spectrometry and thin-layer chromatography (TLC). The UV spectrometric assay was based on the differences in UV spectra of HAP ($\lambda_{max} = 268$ nm), adenine ($\lambda_{max} = 260$ nm), and hypoxanthine ($\lambda_{max} = 250$ nm). Samples were diluted tenfold in 10 mM Tris-HCl (pH 8.0), and UV spectra were recorded on a ND-1000 spectrometer (NanoDrop Technologies) using a ten-fold diluted control sample (no HAP) as a reference. The percent of conversion of HAP to adenine was calculated as $(A_{260}/A_{268} - 0.8545)/0.0042$, the constant values determined with pure solutions containing mixtures of HAP and adenine in various ratios. For TLC, 5 µl of undiluted samples were loaded on Cellulose F plates (Merck). Distilled

water was used as a solvent. The spots were visualized with a UV lamp. In these conditions, R_f values for adenine and HAP were 0.23 and 0.29, respectively.

Acknowledgements

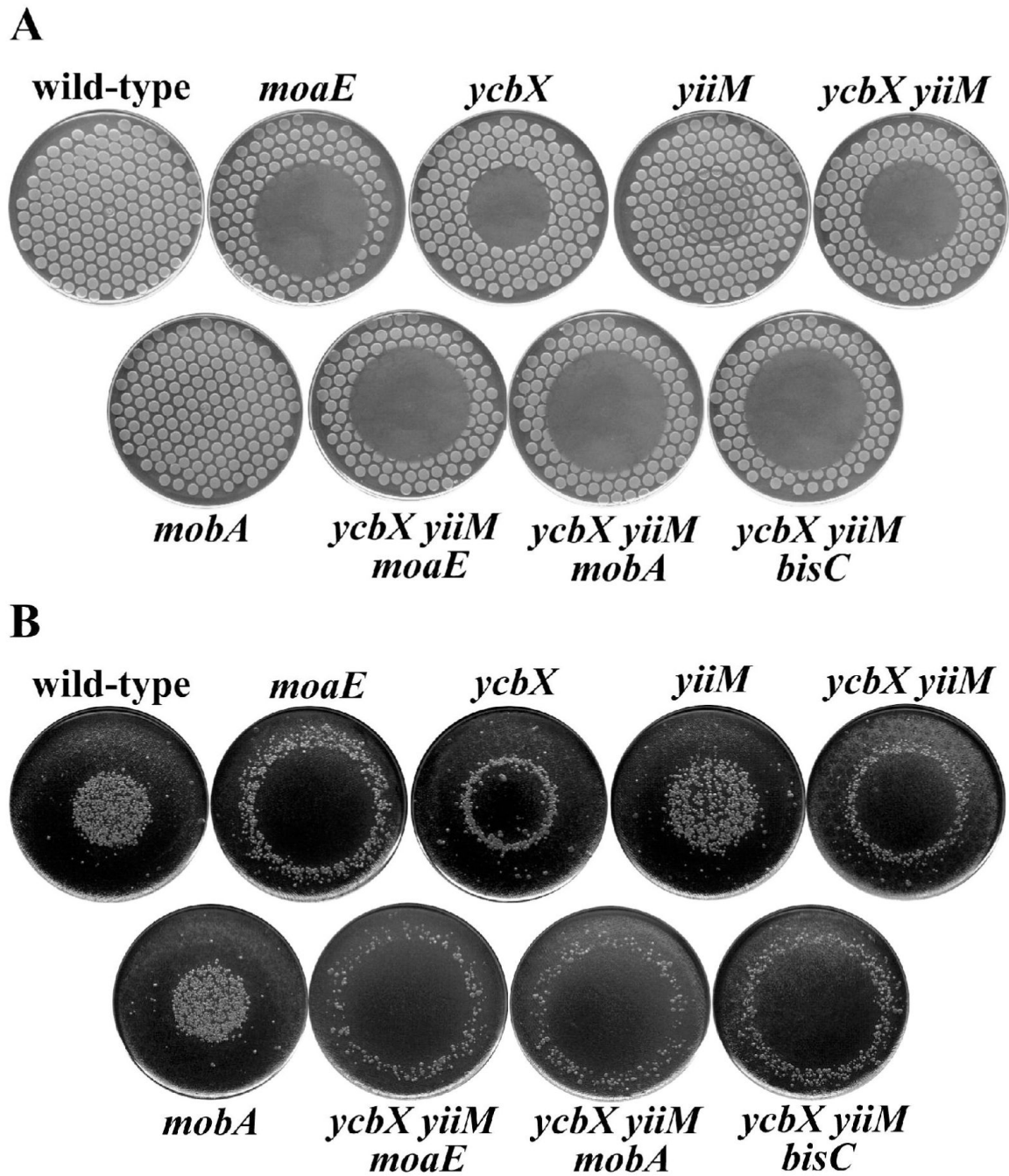
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**Fig. 1.**

HAP-induced killing (A) and mutagenesis (B) for various *E. coli* mutants. The strains used were wild-type (NR10836), *moaE* (NR15996), *ycbX* (NR15873), *yiiM* (NR15871), *ycbX yiiM* (NR15876), *mobA* (NR15994), *ycbX yiiM moaE* (NR16023), *ycbX yiiM mobA* (NR16028), and *ycbX yiiM bisC* (NR16047). Cells were spotted on a minimal medium plate using a multi-prong replicator device, and 50 μ g of HAP was spotted onto the center of each plate (see Experimental procedures). The plates, after overnight incubation, are shown in panel A. These plates were replica-plated onto LB plates containing rifampicin and incubated overnight (panel B).

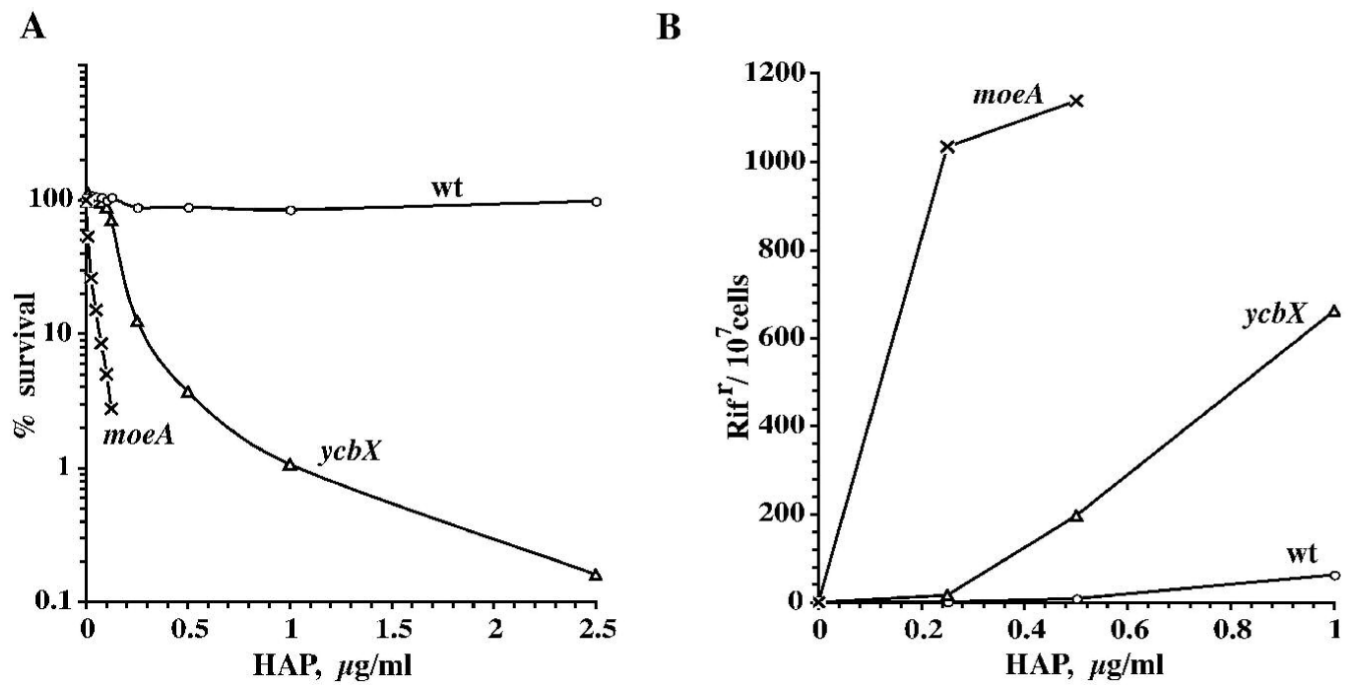


Fig. 2. Survival (A) and mutagenesis (B) of *ycbX* and *moeA* *E. coli* exposed to HAP for 2 h in liquid medium. Strains used were NR10836 (wild-type) (○), NR15873 (*ycbX*::mini-Tn10cam) (Δ), and NR12383 (*moeA121*::mini-Tn10cam) (×). See Experimental procedures for details.

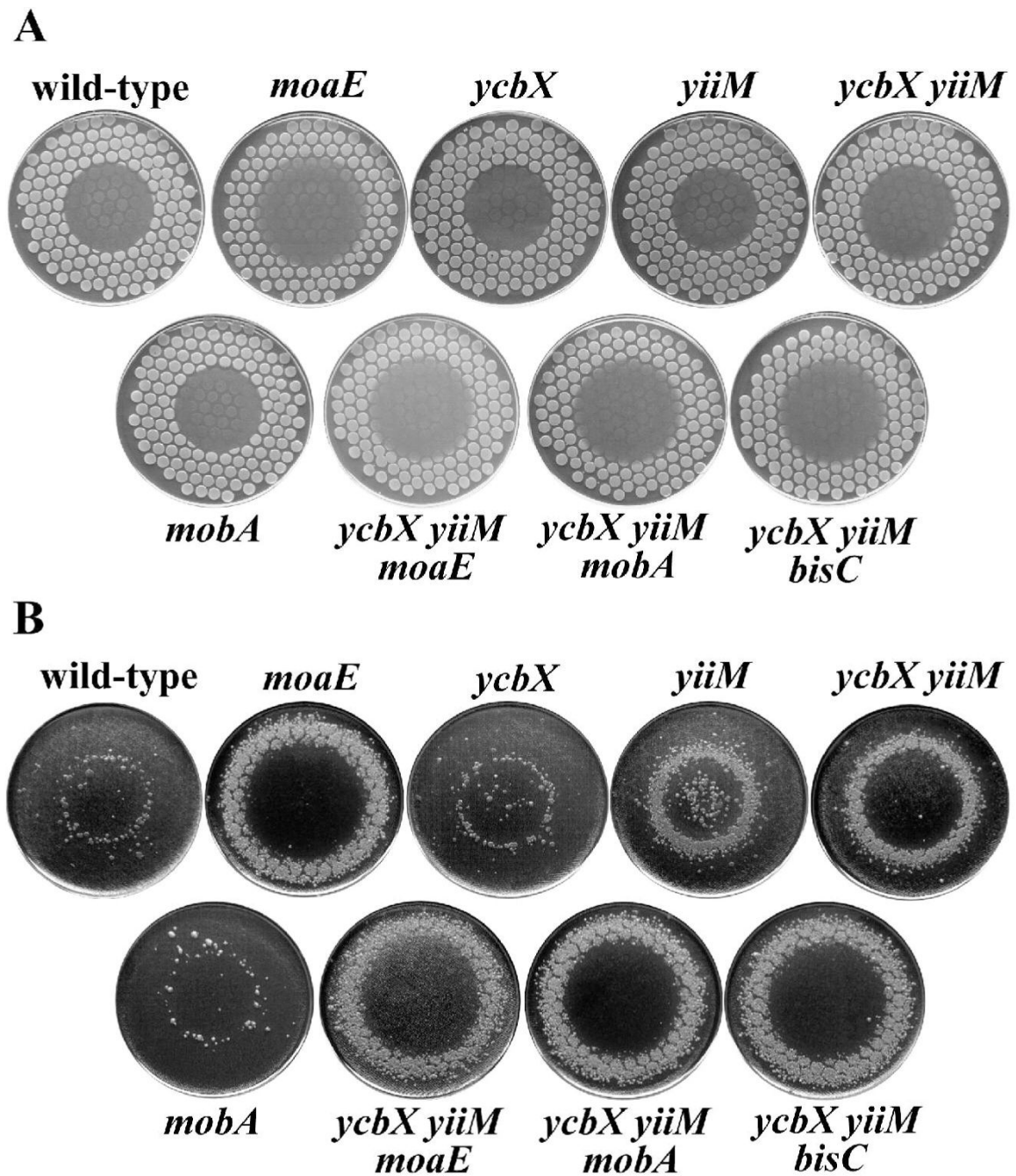


Fig. 3. Spot-test for determination of 2-amino-HAP (AHAP)-induced killing (A) and mutagenesis (B). All strains and procedures were as indicated in the legend to Fig. 1, except that AHAP was used instead of HAP.

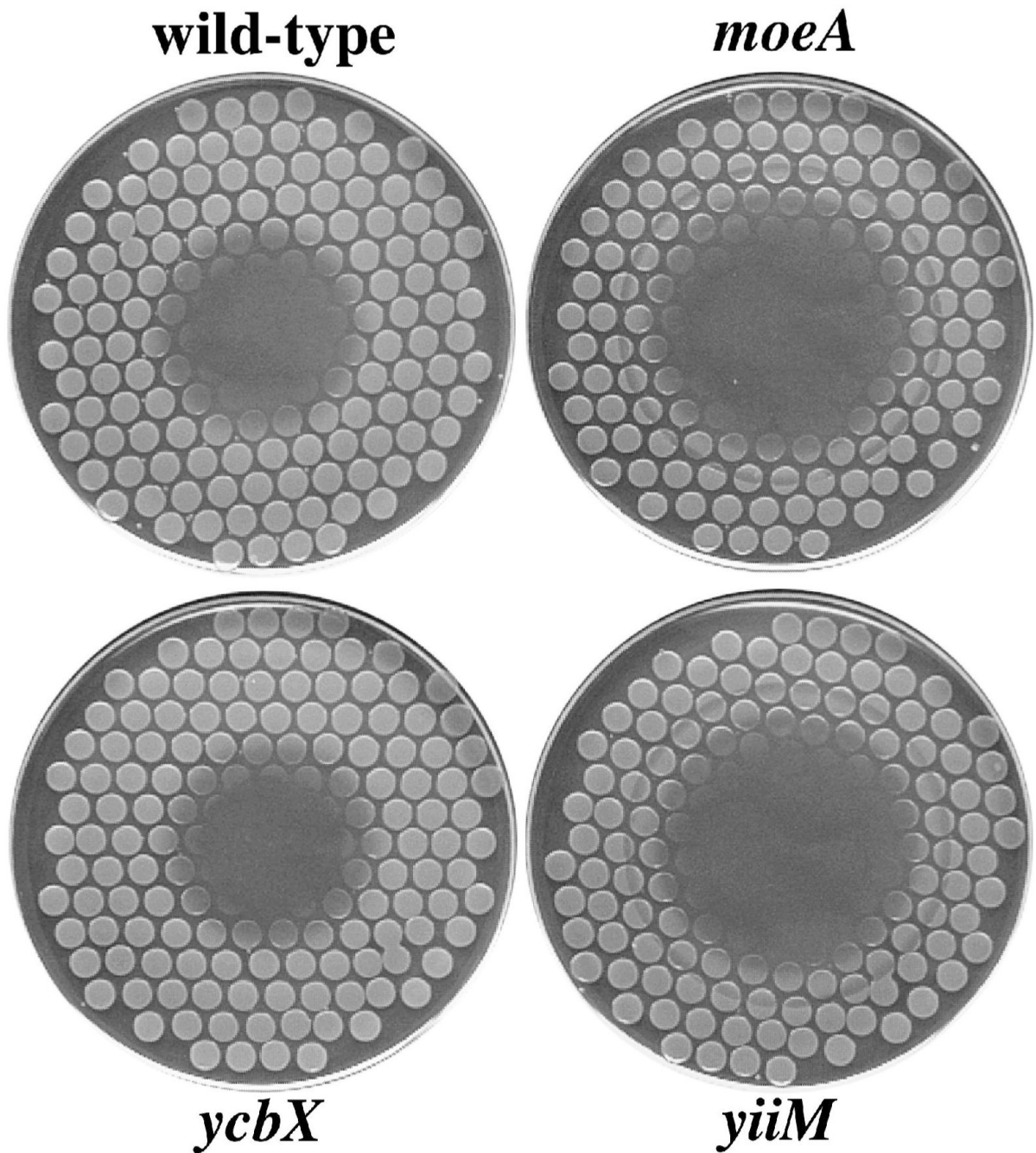


Fig. 4. Sensitivity of *E. coli* strains to hydroxylamine. Cells were plated on minimal medium plates using a multi-prong replicator, and 150 μg of hydroxylamine (in water) was spotted onto the center of each plate. Plates were incubated overnight. See Experimental procedures for details. Strains used were NR10836 (wt), NR12383 (*moeA*), NR15873 (*ycbX*), and NR15871 (*yiiM*). When replicated to LB-Rif plates, no induction of Rif^r mutants was observed for any of the strains (not shown).

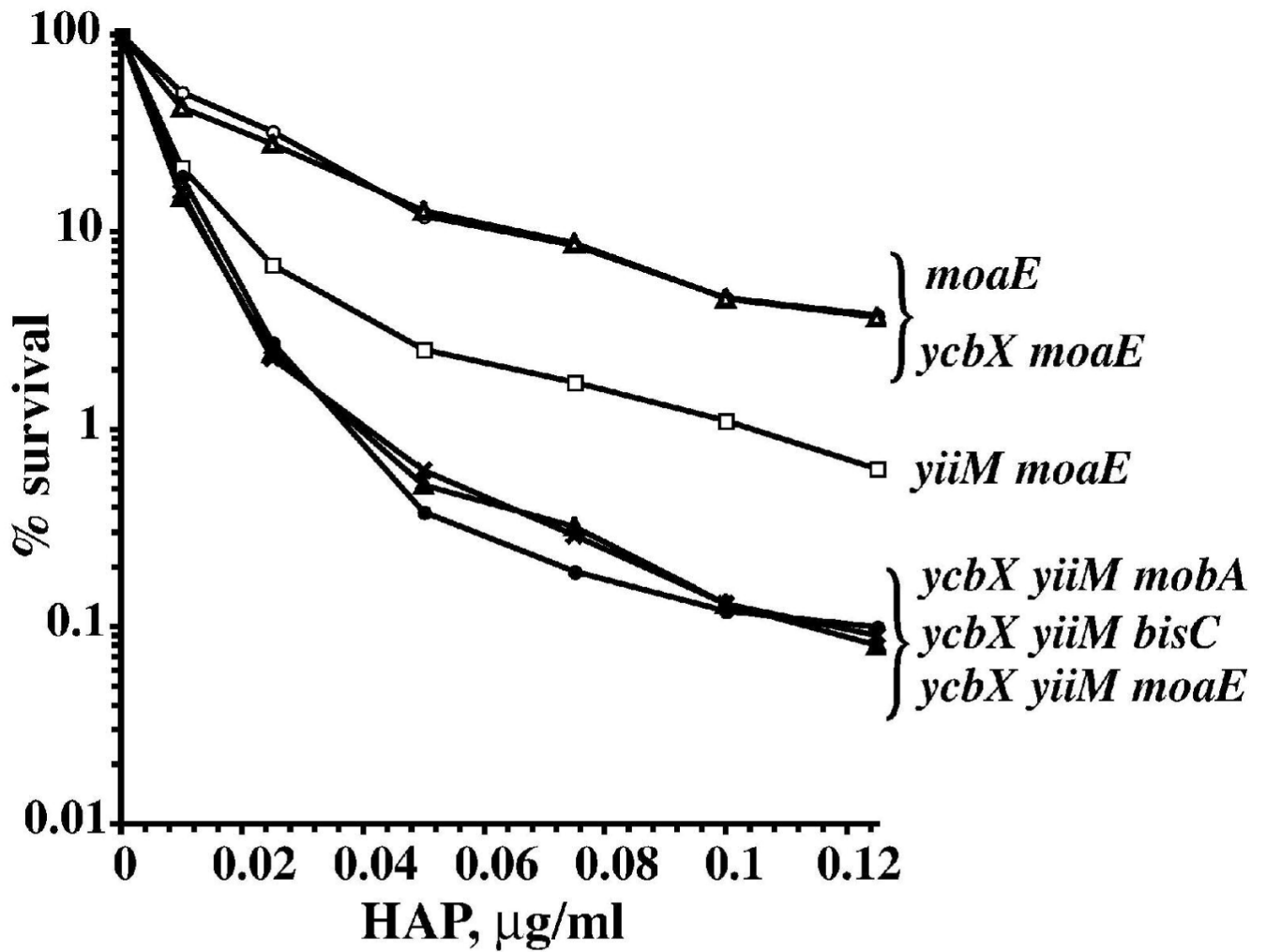


Fig. 5. Role of BisC (biotin sulfoxide reductase) in HAP detoxification. The liquid culture tests were performed as in Fig. 2. The strains used were *moaE* (NR15996) (○), *ycbX moaE* (NR16021) (Δ), *yiiM moaE* (NR16019) (□), *yiiM ycbX mobA* (NR16028) (×), *yiiM ycbX bisC* (NR16047) (σ), and *yiiM ycbX moaE* (NR16023) (λ).

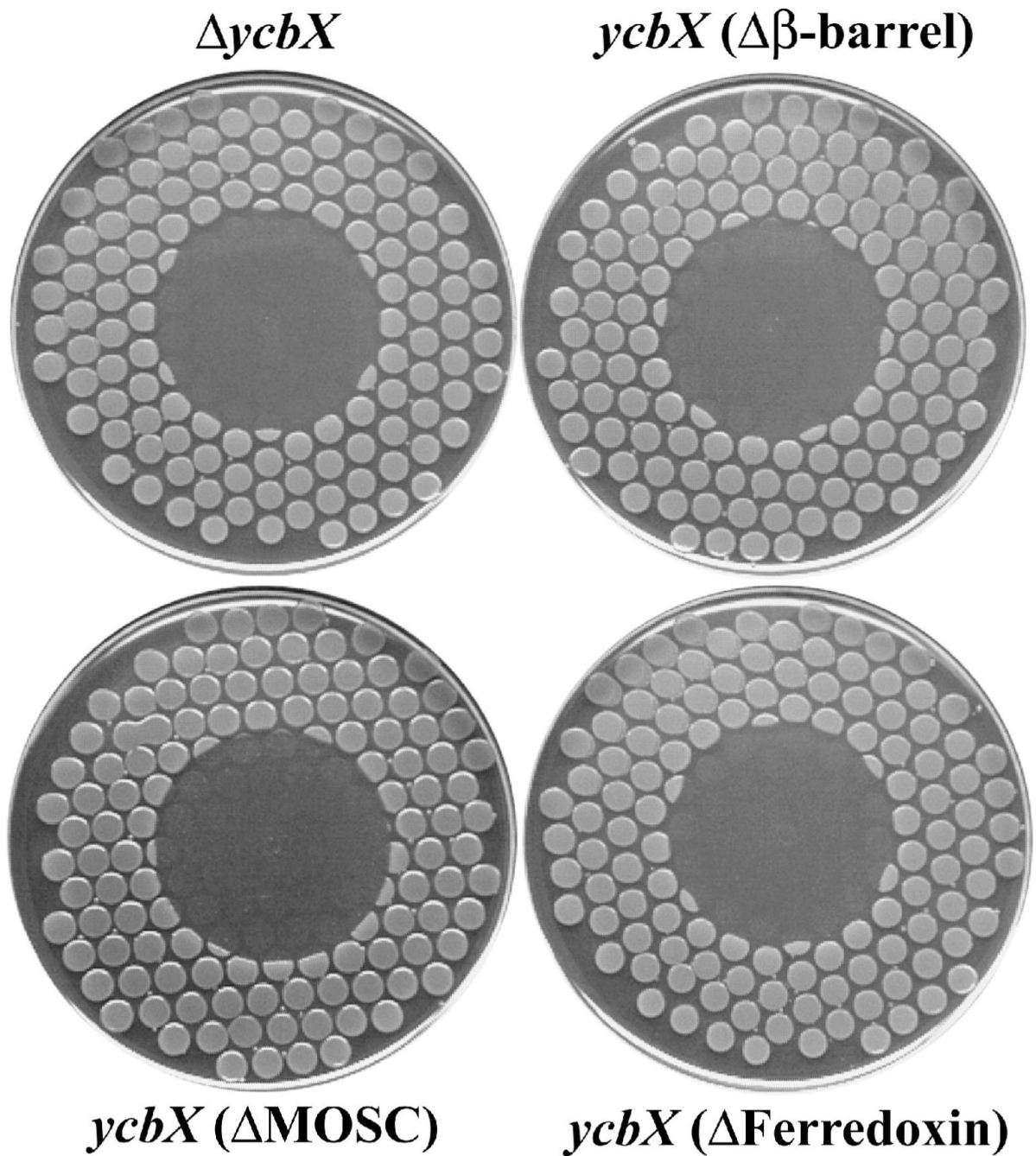
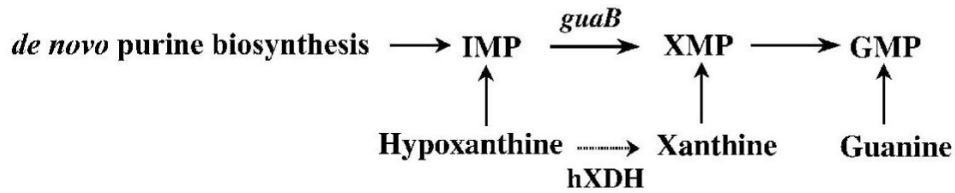


Fig. 7. HAP-sensitivity of strains carrying in-frame deletions of the *ycbX* gene. The *ΔycbX* strain (NR16262) carries a complete deletion of the *ycbX* gene, whereas NR16261, NR16259, and NR16258 encode truncated versions of the YcbX protein lacking, respectively, the β-barrel, MOSC or ferredoxin domain as indicated, and as more fully described in Fig. 6. 50 µg of HAP was spotted onto the center of each plate (see Experimental procedures for more details).

A



B

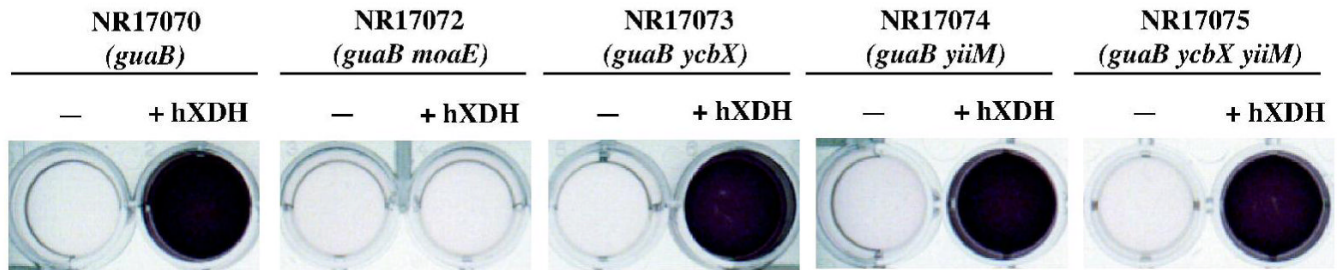


Fig. 8.

In vivo assay for human xanthine dehydrogenase (hXDH) activity in *E. coli*. The cDNA encoding xanthine dehydrogenase was cloned on a pET3a vector, as described in Experimental procedures and tested for its activity in *E. coli* cells. Panel A shows the purine salvage pathways from hypoxanthine, xanthine, and guanine, leading to production of GMP. It also indicates the blockage for usage of hypoxanthine by the *guaB* mutation. The *guaB* block may be relieved by conversion of hypoxanthine to xanthine by hXDH. Panel B shows the results of the experiment. Purple color indicates usage of and growth (respiration) on hypoxanthine as purine source. See text and Experimental procedures for more details.

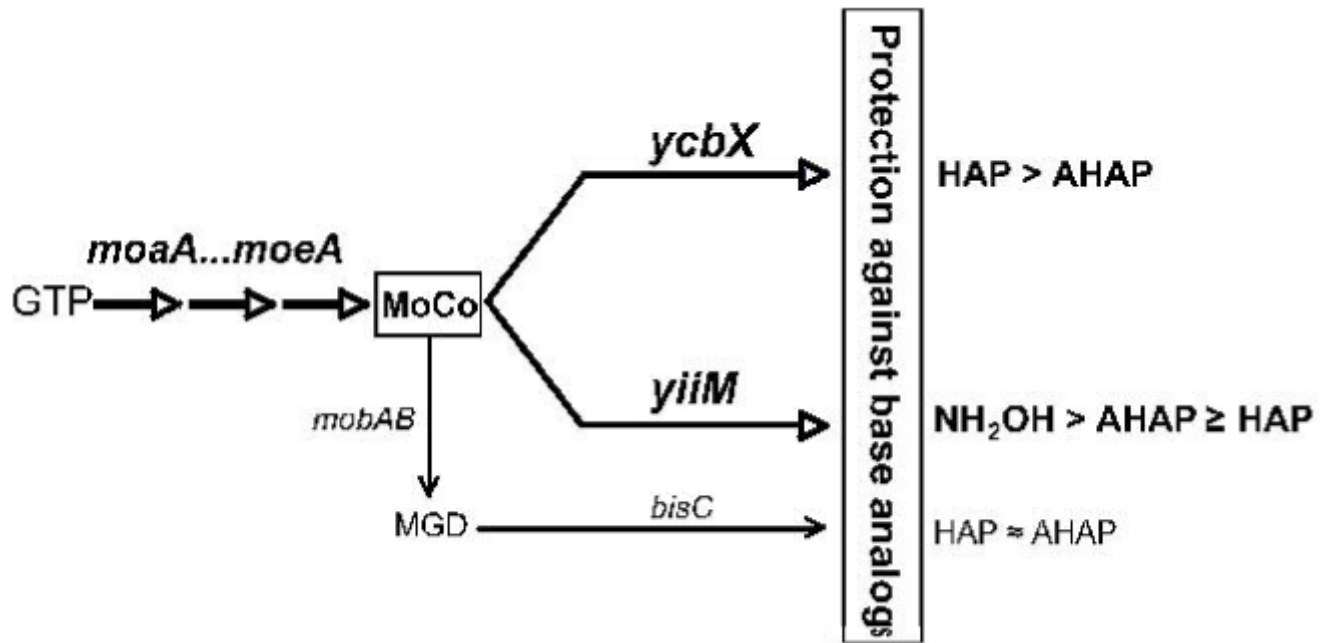


Fig. 9. Genetic framework for pathways of detoxification of *N*-hydroxylated base analogs in *E. coli* based on the current findings. See text for more details.

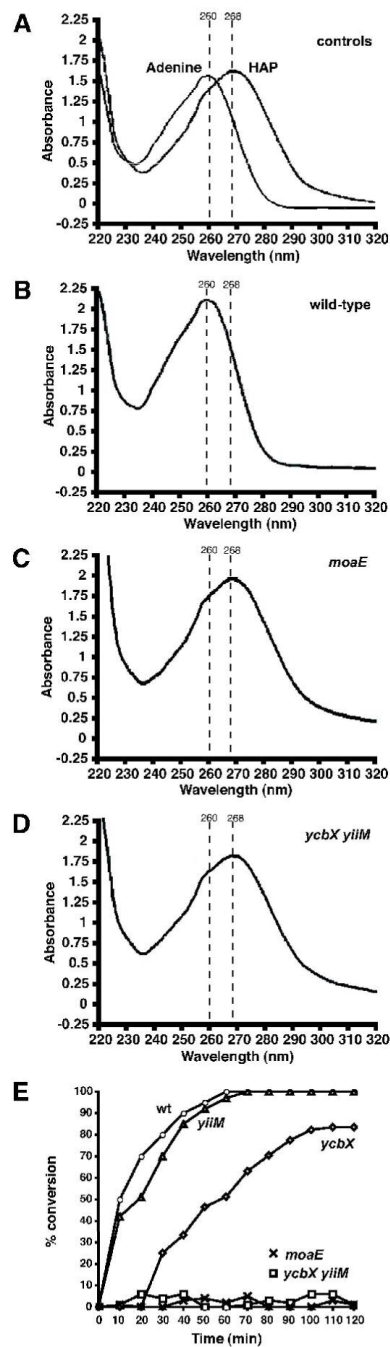


Fig. 10.

HAP conversion to adenine by *E. coli* cell-free extracts. HAP (1 mM) was incubated in extracts prepared from various deamination-deficient (*add ade*) *E. coli*, and the products were analyzed by UV spectra. Panel A shows zero-time controls for either added HAP or adenine, indicating the different peak wavelengths at which the compounds can be detected. Panels B, C, and D show the obtained products after 2 hr incubation of HAP in extracts derived from wt, *moaE*, and *ycbX yjiM* strains, respectively. Panel E shows the time course of conversion of HAP to adenine in the various extracts. See Experimental procedures for details regarding details of extract preparation and sample analysis.

TABLE 1

E. coli strains used in this study

Strain	Genotype	Source or derivation
BL21	<i>ompT hsdS_B(r_B⁻ m_B⁻) gal dcm (Chl^f)</i>	Novagen
BW15113	<i>lacI^f rrmB_{T14} ΔlacZ_{WJ16} hsdR514 ΔaraBAD_{AH33} ΔrhaBAD_{LD78}</i>	Datsenko and Wanner, 2000
KA796	<i>ara thi Δ(pro-lac)</i>	Schaaper <i>et al.</i> , 1985
NR10836	<i>ara thi Δ(pro-lac) F[']CC106</i>	Kozmin <i>et al.</i> , 2000
NR12383	NR10836, but <i>moeA121::mini-Tn10cam</i>	Kozmin <i>et al.</i> , 2000
NR15870	NR10836, but <i>ΔyiiM::kan</i>	This work
NR15871	NR10836, but <i>ΔyiiM</i>	This work
NR15873	NR10836, but <i>yebX::mini-Tn10cam</i>	This work
NR15876	NR10836, but <i>ΔyiiM yebX::mini-Tn10cam</i>	NR15871 × P1/NR15873
NR15967	BL21, but <i>gal⁺, Chl^s</i>	BL21 × P1/NR10836
NR15994	NR10836, but <i>ΔmobA::kan</i>	Kozmin and Schaaper, 2007
NR15996	NR10836, but <i>ΔmoaE::kan</i>	Kozmin and Schaaper, 2007
NR16019	NR10836, but <i>ΔyiiM ΔmoaE::kan</i>	NR15871 × P1/NR15996
NR16021	NR10836, but <i>yebX::mini-Tn10cam ΔmoaE::kan</i>	NR15873 × P1/NR15996
NR16023	NR10836, but <i>ΔyiiM yebX::mini-Tn10cam ΔmoaE::kan</i>	NR15876 × P1/NR15996
NR16028	NR10836, but <i>ΔyiiM yebX::mini-Tn10cam ΔmobA::kan</i>	NR15876 × P1/NR15994
NR16047	NR10836, but <i>ΔyiiM yebX::mini-Tn10cam ΔbisC::kan</i>	This work
NR16048	NR10836, but <i>ΔyiiM yebX::mini-Tn10cam ΔdmsA::kan</i>	This work
NR16049	NR10836, but <i>ΔyiiM yebX::mini-Tn10cam ΔtorA::tet</i>	This work
NR16050	NR10836, but <i>ΔyiiM yebX::mini-Tn10cam ΔtorZ::kan</i>	This work
NR16051	NR10836, but <i>ΔyiiM yebX::mini-Tn10cam ΔnarG::tet</i>	This work
NR16052	NR10836, but <i>ΔyiiM yebX::mini-Tn10cam ΔnarZ::kan</i>	This work
NR16053	NR10836, but <i>ΔyiiM yebX::mini-Tn10cam ΔnapA::kan</i>	This work
NR16054	NR10836, but <i>ΔyiiM yebX::mini-Tn10cam ΔfdnG::kan</i>	This work
NR16055	NR10836, but <i>ΔyiiM yebX::mini-Tn10cam ΔfdhF::kan</i>	This work
NR16056	NR10836, but <i>ΔyiiM yebX::mini-Tn10cam ΔfdoG::tet</i>	This work
NR16057	NR10836, but <i>ΔyiiM yebX::mini-Tn10cam ΔynfE::kan</i>	This work
NR16058	NR10836, but <i>ΔyiiM yebX::mini-Tn10cam ΔynfF::kan</i>	This work
NR16060	NR10836, but <i>ΔyiiM yebX::mini-Tn10cam ΔydeP::kan</i>	This work
NR16258	NR10836, but <i>ΔyebX[Δ(Ferredoxin)]</i>	This work
NR16259	NR10836, but <i>ΔyebX[Δ(MOSC)]</i>	This work
NR16261	NR10836, but <i>ΔyebX[Δ(β-barrel)]</i>	This work
NR16262	NR10836, but <i>ΔyebX[Δ(β-barrel, MOSC, Ferredoxin)]</i>	This work
NR17070	NR15967, but <i>λ(DE3) ΔmobA ΔguaB::tet</i>	This work
NR17072	NR17070, but <i>ΔmoaE::kan</i>	NR17070 × P1/NR15996
NR17073	NR17070, but <i>yebX::mini-Tn10cam</i>	NR17070 × P1/NR15873
NR17074	NR17070, but <i>ΔyiiM::kan</i>	NR17070 × P1/NR15870
NR17075	NR17070, but <i>ΔyiiM::kan yebX::mini-Tn10cam</i>	NR17074 × P1/NR15873
NR17315	BW25113, but <i>ΔyebX::kan</i>	This work

Strain	Genotype	Source or derivation
NR17326	NR10836, but $\Delta add \Delta ade::cam$	This work
NR17330	NR17326, but $\Delta yiiM$	NR17070 \times P1/NR15870 and elimination of <i>kan</i>
NR17485	NR17326, but $\Delta moaE::kan$	NR17326 \times P1/NR15996
NR17486	NR17326, but $\Delta ycbX::kan$	NR17326 \times P1/NR17315
NR17487	NR17326, but $\Delta yiiM \Delta ycbX::kan$	NR17330 \times P1/NR17315

Details about the various strain constructions can be found in Experimental procedures.

Ch^I and Ch^S indicates chlorate resistance and sensitivity, respectively. The $\Delta ycbX::kan$ present in NR17315 is the complete deletion $\Delta ycbX\Delta(\beta\text{-barrel, MOSC, Ferredoxin})::kan$.