A genetic sensor for strong methylating compounds

Felix Moser[1],[*], **Andrew Horwitz**[2],[*], **Jacinto Chen**[2], **Wendell A. Lim**[2], and **Christopher A. Voigt**[1],[†]

[1]Synthetic Biology Center, Department of Biological Engineering, Massachusetts Institute of Technology, Boston, MA 02139, USA

[2]Howard Hughes Medical Institute and Department of Cellular and Molecular Pharmacology, University of California - San Francisco, San Francisco, CA 94158, USA

Abstract

Methylating chemicals are common in industry and agriculture and are often toxic, partly due to their propensity to methylate DNA. The *Escherichia coli* Ada protein detects methylating compounds by sensing aberrant methyl adducts on the phosphoester backbone of DNA. We characterize this system as a genetic sensor and engineer it to lower the detection threshold. By overexpressing Ada from a plasmid, we improve the sensor's dynamic range to 350-fold induction and lower its detection threshold to 40 μM for methyl iodide. In eukaryotes, there is no known sensor of methyl adducts on the phosphoester backbone of DNA. By fusing the N-terminal domain of Ada to the Gal4 transcriptional activation domain, we built a functional sensor for methyl phosphotriester adducts in *Saccharomyces cerevisiae*. This sensor can be tuned to variable specifications by altering the expression level of the chimeric sensor and changing the number of Ada operators upstream of the Gal4-sensitive reporter promoter. These changes result in a detection threshold of 28 μM and 5.2-fold induction in response to methyl iodide. When the yeast sensor is exposed to different S_N1 and S_N2 alkylating compounds, its response profile is similar to that observed for the native Ada protein in *E. coli*, indicating that its native function is retained in yeast. Finally, we demonstrate that the specifications achieved for the yeast sensor are suitable for detecting methylating compounds at relevant concentrations in environmental samples. This work demonstrates the movement of a sensor from a prokaryotic to eukaryotic system and its rational tuning to achieve desired specifications.

Keywords

synthetic biology; genetic circuit; genetic device; fumigant; Gal4

[†]corresponding author: NE47-277, 500 Technology Square, Cambridge, MA, 02139, cavoigt@gmail.com.
[*]These authors contributed equally to this work.

Introduction

A transcriptional genetic sensor is a unit of DNA that contains all of the necessary parts to convert an input stimulus to the up- or down-regulation of a promoter[1,2]. Following this paradigm, the output promoter of a sensor can be used as the input promoter of a genetic circuit, which can implement signal-processing functions. Genetic sensors have been constructed that respond to many environmental signals, including light[3,4], temperature[5,6], gases[7,8], toxins (*e.g.*, arsenic)[9,10], and chemicals (*e.g.*, industrial products, pollutants or explosives)[11,12,13,14]. Many of these sensors are based on the transfer of parts from one organism to another; for example, moving a TNT sensor from *E. coli* to *Arabidopsis*[13], an artificial quorum sensing system made of *Arabidopsis* parts transferred to yeast[15], light sensors from cyanobacteria and plants to *E. coli* and mammalian cells[3,16], and a redox sensor from *Streptomyces* to mammalian cells[17]. Such transfers often require sensor re-engineering and the substitution of parts to make the sensor functional in the new host.

Different applications require different performance specifications of a genetic sensor, which can be achieved by tuning the response function of the sensor. The response function is defined by how the sensor output (promoter transcription) changes as a function of the input stimulus. The shape of this function captures the responsiveness of the sensor to the input and provides information that aids its connection to a downstream circuit[18,19]. There are several descriptors of the response function that are particularly useful: the basal activity, cooperativity, dynamic range, detection threshold (lowest input concentration sensed above background), and sensitivity (the slope during the transition)[20]. Additionally, it is useful to determine the specificity of a sensor to understand how a sensor will respond in a mixture of ligands or complex environment. Various approaches, including directed evolution, have been applied to alter the properties of genetic sensors[21,22]. Synthetic biology has also developed "tuning knobs" to control transcription and translation that could be applied to altering sensor response[23,24, 25,26,27].

Here, we design and characterize a sensor of methylating compounds, transfer it from *E. coli* to yeast, and tune its response characteristics. Methylating agents are relevant to human health because they can induce the aberrant methylation of DNA, which can lead to mutations, misregulation, and ultimately disease. Many methylating agents leave methyl phosphotriester (PTE) adducts on DNA. These adducts are very stable, long-lasting moieties in eukaryotic cells due to their innocuous nature and resistance to DNA repair[28]. Because of their stability, methyl PTE adducts have been proposed as a biomarker for cumulative genotoxic exposure[29,30]. Methylating agents that generate these adducts are common in industrial and agricultural processes and often produce other more damaging DNA lesions. For example, phenyl glycidyl ether (PGE) and *N*-dimethylnitrosoamine (NDMN) are used in the manufacture of paint, resin and rubber[31,32]. Dimethyl sulfate (DMS) is a common alkylating agent used in kiloton quantities in a variety of industries[33]. Methylnitronitrosoguanidine (MNNG) and methyl methanesulfonate (MMS) are used in laboratories to study DNA damage and repair[34]. Methyl halides such as methyl chloride and methyl iodide (MeI) are methylating agents that are being controversially used as soil fumigants and intermediates to various chemical processes, including silicon rubber production[35,36]. All of these agents methylate the bases of DNA[55]. Due to the ubiquity and potency of genotoxic methylating agents, a sensor for DNA methylation damage could be a tool for environmental biosensing or a diagnostic system for long-term genotoxic exposure.

Our lab previously engineered *E. coli* and *S. cerevisiae* to produce methyl halides by introducing a methyl halide transferase (MHT) gene[37]. Screening for MHT activity is tedious and low throughput because it is based on a GC-MS assay. Cell-based sensors have been used as a tool to screen libraries of mutant enzymes and pathways for increased activity

or titer[38,39]. To facilitate easier screening of MHT activities, we aimed to develop a sensor for methyl halide production in *Saccharomyces cerevisiae*, the organism in which the greatest MeI yield was achieved.

Escherichia coli has a strong adaptive response to methylating agents such as methyl halides. These agents are sensed via the Ada protein, which is either directly methylated by S_N2 methylating agents or indirectly by S_N1 methylating agents via methyl PTE DNA adducts[40,41,42,43]. Ada moves along DNA, detects, and then transfers a single DNA Sp methyl PTE adduct onto its Cys38 residue (Figure 1A). The methylation of Ada's Cys38 residue activates Ada as a transcription factor. Ada then upregulates transcription of various DNA repair proteins, including its own *ada* gene. This positive feedback loop turns the very low basal expression of Ada into a strong, sustained response to the exposure of genotoxic methylating agents[44]. Ada has been used as a sensor for DNA methylation toxicity of genotoxic compounds to complement the Ames test, the gold standard for assaying mutagenicity of a compound[45].

No comparable, specific sensor of DNA methyl PTEs is known in eukaryotes. To develop such a sensor in eukaryotes, we fused the N-terminal domain of Ada (N-Ada) to the Gal4 transactivation domain. This Gal4 domain, taken from yeast, is functional in a broad range of hosts, including yeast, flies, plants, and human cells[46,47,48]. We demonstrate that the resulting Gal4-N-Ada fusion protein acts as a specific and strong sensor of methylating compounds in *Saccharomyces cerevisiae*. We show that the sensor retains Ada's characteristic specificity for methylating compounds, indicating that Gal4-N-Ada can detect and remove DNA methyl PTE adducts in *S. cerevisiae*. To demonstrate tuning the *S. cerevisiae* sensor to different specifications, we change the detection threshold of the sensor by changing expression of the sensor protein and change its sensitivity by altering the number of operators in the promoter driving the reporter. Finally, we demonstrate the utility of the tuned *S. cerevisiae* sensor to detect MeI in an MHT-expressing culture and in a complex soil sample.

Results

Construction of a methylation sensor in E. coli

The native *E. coli ada* promoter was used to measure the sensor response to methylating compounds. The *ada* promoter region, which includes a single Ada operator upstream of the −45 site, was transcriptionally fused to a green fluorescent protein (GFP) reporter on a p15A plasmid backbone (Figure 1A). In the first design, Ada is expressed from its native locus in the *E. coli* MG1655 genome. When uninduced, it has been estimated that there are 2–4 Ada proteins per cell[49]. Upon induction with MeI, this sensor shows a strong 250-fold activation and detection threshold of 100 μM MeI (Table 1; Figure 1B). Near the switch point of the response function, the population of cells exhibits a bimodal distribution of fluorescence (Figure 1B, Figure S1). This is characteristic of positive feedback loops, as in the case of the native autoregulatory control of Ada expression.

A challenge in the design of genetic sensors is the tuning of their detection threshold to respond to different target levels of stimulus. To this end, we sought to lower the detection threshold of the Ada sensor to respond to lower concentrations of MeI. This was achieved by increasing the expression level of the Ada protein. A plasmid was constructed in which the *ada* gene was placed under control of the arabinose-inducible P_{BAD} promoter on a low-copy incW origin plasmid. Even in the absence of inducer, the basal expression of Ada from P_{BAD} lowered the detection threshold of the sensor and increased its dynamic range (Table 1; Figure 1C). When the Ada concentration was further increased via arabinose induction, the detection threshold decreased from 100 μM to 6 μM. At intermediate levels of Ada, the

OFF state of the sensor stayed at a constant level. However, when Ada was maximally expressed from P_{BAD} (10 mM arabinose) the basal activity of the OFF state increased significantly, which attenuated the dynamic range of the sensor.

The impact of knocking out the native *ada* gene on the sensor was investigated. This knockout eliminated the positive feedback loop. As expected, the sensor was non-responsive to MeI when *ada* is knocked out (Figure 1D). This response was rescued when Ada was expressed from P_{BAD}. The detection threshold of the sensor was similar to when Ada was genomically expressed. However, the response function was impacted in several ways that are consistent with the disruption of a positive feedback loop[50]. First, the cooperativity of the response function decreased significantly (Table 1, Figure 1B), making the sensor less sensitive to changes in MeI near the threshold. Second, the highest ON state of the sensor depended more on the level of Ada expression, increasing by 10.7-fold from basal expression to full induction of Ada. The bimodality of the response was also disrupted, which diminished the variability in the population near the switch point (Figure S2). These are frequently desirable properties because the analog behavior, broad induction range, and cell uniformity are useful for creating quantitative assays[51].

Construction of a methylation sensor in S. cerevisiae

To move the Ada sensor into yeast, we built a chimeric protein that contains the N-terminal domain of Ada (N-Ada, residues 1 to 180) fused to the Gal4 trans-activator (residues 767 to 881; Figure 1E). N-Ada is the site of DNA binding and methyltransferase activity and is necessary and sufficient to induce the adaptive response in *E. coli*[41]. The Gal4 trans-activation domain is a native yeast protein that upregulates transcription when localized to the P_{Cyc1} promoter[52]. We modified the P_{Cyc1} promoter to include 8 Ada operators ($P_{8x.Cyc1}$) and placed it upstream of an enhanced GFP (EGFP) reporter (Figure 1E). The strong, constitutive P_{Adh1} promoter was placed upstream of the Gal4-N-Ada chimera. A *S. cerevisiae* strain was built based on the completed sensor ($P_{8x.Cyc1}|P_{Adh1}$) by integrating the P_{Adh1}-driven Gal4-N-Ada expression cassette and the $P_{8x.Cyc1}$-driven EGFP reporter cassette into the genome (Materials and Methods).

We hypothesized that exposure of this strain to methylating agents would lead to methylation and subsequent activation of the N-Ada domain. This would localize the Gal4-N-Ada fusion protein to $P_{8x.Cyc1}$ and upregulate expression of the EGFP reporter. Upon exposure to MeI, the completed yeast sensor strain $P_{8x.Cyc1}|P_{Adh1}$ showed a maximal 5.2-fold induction of the EGFP reporter (Figure 1F). The population's fluorescence changed gradually with the concentration of MeI and no bimodality in the population's fluorescence distribution was observed (Figure S2). Additionally, the response function was more linear than the native system in *E. coli* (Table 2). Both of these observations are consistent with the response observed when the positive feedback loop in *E. coli* is disrupted (Figures 1D and S1). The detection threshold of this yeast sensor to MeI is 28 μM, which is lower than the uninduced *E. coli* sensors (Table 2).

In building the sensor, variations of the P_{Cyc1} promoter containing different numbers of Ada operators were tested (Figure 1G and S2). The level of expression from a Gal4-driven promoter is a function of how many Gal4-containing proteins are recruited to the promoter[53]. Therefore, increasing the number of operators upstream of the target promoter can tune the response of the sensor. Variations of the P_{Cyc1} promoter containing 0, 1, 3, and 8 copies of the Ada operator were built. The presence of one copy of the operator upstream of P_{Cyc1} was sufficient to upregulate transcription from the promoter, even in the uninduced state (Figure 1G). Both the dynamic range and sensitivity increased when 8 operators were included in the promoter, but no significant difference was observed between 1 and 3 operators (Table 2). The detection threshold of the sensor did not change with the number of

operators in the P_{Cyc1} promoter, and no significant increase in cooperativity was observed when the number of operators was increased (Table 2). These results show that changing the number of operators driving the P_{Cyc1} promoter enables tuning of the dynamic range and sensitivity of the response.

The impact of varying the expression level of Gal4-N-Ada was also tested. The sensor uses a constitutive promoter to drive the expression of Gal4-N-Ada. When the P_{Adh1} promoter is used, the detection threshold is 28 μM MeI and is independent of the number of operators. We hypothesized that, similar to the Ada sensor in *E. coli*, the detection threshold was dependent on the level of Gal4-N-Ada expression. To test this, we replaced P_{Adh1} with the 20 to 100-fold weaker P_{Cyc1} promoter[54]. This replacement resulted in a 10-fold higher detection threshold of 340 μM (Figure 1H). Notably, this change in the threshold did not affect the magnitude of the ON or OFF states.

Comparison of sensor responses to different methylating compounds

To test whether the Gal4-N-Ada sensor retained its native activity following species transfer, both the *E. coli* and yeast methylation sensors were exposed to a panel of different S_N1 and S_N2 alkylating agents. S_N1 and S_N2 agents react via different mechanisms and have different affinities for methylating DNA[43,55]. The specificity of the yeast methylation sensor's response to these agents was expected to be comparable to the *E. coli* sensor.

S_N1 agents, such as MNNG, are known to promiscuously methylate the phosphoester backbone of DNA and have not been observed to methylate Ada directly[40,43]. In nature, nitrosoamines similar to MNNG are produced via endogenous chemistry and are thought to be the source of naturally occurring DNA methyl PTE adducts[30]. As such, MNNG is highly toxic to both organisms (Figure S4). The *E. coli* and yeast sensors both responded strongly to MNNG, showing the lowest observed detection thresholds (Figure 2A, Table 3). Because MNNG is only known to activate Ada indirectly through methylation of DNA, this supports the hypothesis that Gal4-N-Ada is detecting and removing methyl PTE adducts from the DNA backbone. Interestingly, the *E. coli* sensor is less cooperative in its response to MNNG as compared to MeI and other S_N2 compounds (Table 3).

S_N2 agents such as MeI, MMS, and DMS readily activate Ada in *E. coli* (Figures 1 and 2). Though these agents have not been observed to attack the phosphoester backbone[43,56] of DNA, MeI has been observed to methylate Ada directly *in vitro*[40]. It is not known to what extent S_N2 agents activate Ada directly or indirectly via scant DNA phosphoester methylation. The yeast sensor responded to all of the S_N2 methylation agents. Compared to the *E. coli* sensor, it responded to MMS and DMS with a lower detection threshold and a more graded, less cooperative response (Table 3). DMS can donate two methyl groups and is more toxic than MMS (Figure S4). Both sensors detected DMS at lower concentrations than MMS (Table 3).

Ada is also sensitive to the size of the alkyl group of PTE adducts on the DNA backbone. Larger alkyl groups sterically hinder the mechanism of detection and activate Ada poorly[57,58]. EMS, an analogue of MMS that donates a larger ethyl group, was added to the *E. coli* and yeast sensors to test for the retention of this specificity. As expected, neither sensor responded to EMS (Figure 2D). The fact that the yeast sensor responded to the same range of alkylating agents as the native *E. coli* sensor suggests that the Gal4-N-Ada sensor retains much of its native activity in yeast, including its ability to detect and remove methyl PTE adducts on DNA.

Biosensing applications

We previously reported the construction and screening of a library of 70 homologous methyl halide transferases (MHTs)[37] and subsequent engineering of an MHT-expressing *S. cerevisiae* strain for production of high titers of MeI. To enable faster screening of MHT libraries and further engineering of productive yeast strains, we sought to use the yeast methylation sensor as a reporter of MHT activity. Different MHT enzymes have been shown to produce 0.3 – 1.3 mM MeI / hr in *S. cerevisiae*[37]. Because this range is consistent with the thresholds obtained for the genetic methylation sensors, we predicted that it would be possible to use them as a cell-based screen.

We carried out an experiment to determine if the methylation sensor could respond to MeI produced in yeast and whether the linear range is sufficient to distinguish enzymes of different activity. For this experiment, the *S. cerevisiae* $P_{8x.Cyc1}|P_{Adh1}$ sensor strain was transformed with plasmids encoding a set of 7 MHT homologues (Figure 3A). Each strain was then grown to high density and MeI production was induced by adding NaI (Methods). The cells were grown for 1 hour, after which each culture was analyzed by cytometry and the MeI titer was measured by analyzing the headspace using GC-MS. The sensor output correlated with the activities produced by the different MHT homologues and saturated at high titers (Figure 3A).

Another potential application for the genetic sensor is as a biosensor for environmental samples. In particular, methyl bromide and methyl iodide are used in agriculture as soil fumigants. MeI is typically used at initial concentrations of 0.4 – 0.6 mM during fumigation[59] but dissipates quickly due to evaporation and subsequent light-induced decay. However, decay rates vary with soil composition, and MeI can be found in some soils for up to several days after exposure[60,61]. On-site measurement of MeI levels with advanced instrumentation is impractical. The development of biosensors for fast, cheap on-site detection of compounds is a valuable alternative[62].

We sought to assess the sensor's utility as a biosensor for the presence of MeI in soil. To test this, we added an aqueous solution of MeI to soil and then monitored MeI levels in the soil over time using the yeast $P_{8x.Cyc1}|P_{Adh1}$ sensor (Methods). At different time points, the soil samples were fractionated by centrifugation and the runoff was collected. The runoff was then added to the culture and grown for three hours, after which the cells were assayed by cytometry. Due to reaction and evaporation, MeI is lost exponentially from the soil ($t_{1/2}$ = 1.5 – 2.0 hours), which is consistent with the degradation of MeI in soils with high organic content[61]. To control for MeI loss due to evaporation from the soil sample, half the sample tubes were closed during the assay, but this was found to have minimal impact on sensor activity. The sensor showed no activation when MeI was omitted, indicating that it responded specifically to the MeI present in the soil runoff. Because soil runoff is a complex mixture of compounds, this also demonstrated the sensor's specificity and robustness.

Discussion

Our results demonstrate that the Ada methylation sensor is functional after its transfer from *E. coli* into yeast. Additional engineering was required to convert the Ada response into a transcriptional signal. MNNG, an S_N1 methylating agent, is only known to activate Ada indirectly by methylating the phosphoester backbone of DNA. The S_N2 reagents MeI, MMS, and DMS are hypothesized to methylate Ada's Cys38 residue either directly or indirectly via undetectable amounts of methylation of the PTE backbone of DNA. The fact that the yeast sensor responded to the entire array of methylating agents supports the hypothesis that the N-terminal domain of Ada retained its native functions in the transfer from *E. coli* to yeast and that it detects and removes methyl PTE adducts from eukaryotic

chromosomal DNA. We know of no other system for the detection or removal of DNA methyl PTE adducts in eukaryotic cells. It should be noted that while the sensor is operational in yeast, there was no impact on resistance to methylating toxins (Figure S4).

Several tuning strategies were effective at changing the performance of the sensors in both organisms. Tuning the expression of the sensor protein consistently changed the detection threshold. However, in *E. coli* this came with a tradeoff where at very low detection thresholds the basal activity of the sensor increased. This high basal activity also occurred for the engineered sensor in yeast and was constant irrespective of the strength of Gal4-N-Ada expression. We also found that the dynamic range and sensitivity of the yeast sensor could be improved by increasing the number of Ada operators in the output promoter, but this effect attenuates after 3 operators, consistent with previous observations[63]. Although the yeast sensor was unable to achieve the same response as observed in the native *E. coli* system, several untried strategies are available to further optimize the sensor. The target P_{Cyc1} promoter may be improved by weakening its basal activity or optimizing the spacing of the Ada operators. The Gal80 transcription factor, which inhibits Gal4 activity and was present in all our strains, may be deleted from the genome to improve Gal4 activity[64].

Additional network architectures can impact sensor performance. The native *E. coli* Ada system contains a strong positive feedback loop that amplifies a basal state of only 2–4 Ada proteins per cell[43] to one of thousands of Ada proteins per cell[65]. This has been shown to result in a more ultrasensitive, digital response[66]. An ultrasensitive response could be replicated in yeast by either building such a loop or through the inclusion of interactions that sequester the regulator[67,68]. On the other hand, to engineer a more linear, faster, or pulsed response in the sensor, one could implement negative genetic feedback[69,70,71]. More complex architectures, such as feed-forward loops, can also be used to engineer complex dynamics and robustness to noise[72,73]. Also useful for sensing systems would be the engineering of "scale-free sensing", a characteristic of some complex networks that enables those networks to detect changes in the environment regardless of the level of the background signal[74,75]. Clearly, many modes of action remain open to further engineer the sensors presented here to altered dynamics and specifications.

A persistent challenge in synthetic biology has been the development of well-characterized sensors that can respond to environmental signals. The design of a functional sensor and its tuning to a particular performance specification is often more difficult than building genetic circuits, in part because a ligand-binding event has to be converted into a transcriptional output. The movement of sensors between organisms has provided a successful avenue for the production of novel sensors. However, such movement often requires extensive re-engineering of a sensor and can be prone to unpredictable context effects[76,77]. Sensors for strong methylating compounds present a novel sensory input that can be harnessed in genetic engineering. Beyond the applications outlined in this paper, the MHT enzymes and Ada sensor also offer new parts that can have other uses. For example, these modules may act as sensor and receiver devices for engineering communication between cells where the volatile methyl halide signal acts in the gas phase. Also, the slow decay and orthogonality of the methyl PTE adducts of DNA in eukaryotes may enable a route to engineering epigenetic memory, though this application will be limited by the dilution of the methyl PTE adducts by half with each successive generation. The specific parts from this study as well as the generalizable design and tuning strategies can be broadly applied to problems in design, species-transfer, and tuning of novel genetic sensors.

Materials and Methods

Strains and Media

Cloning was performed in *E. coli* DH10B and plasmids were transformed into *E. coli* MG1655 or *E. coli* MG1655Δ*ada* for measurement. *E. coli* transfer function assays were performed in 1 ml of supplemented M9 media, containing 0.2% casamino acids (BD #228820), 1 mM thiamine HCl (Sigma-Aldrich T4625), and antibiotics. The *E. coli* MG1655Δ*ada* strain was made by deleting the *ada* CDS (2307363..2308427) from the *E. coli* MG1655 chromosome using the technique of Datsenko and Wanner[78]. To maintain plasmids in *E. coli*, we used antibiotic concentrations of 100 μg/ml for kanamycin and 100 μg/ml for spectinomycin. For all yeast experiments, we used *S. cerevisiae* strain SO992 (W303-derived, *MATa, trp1, leu2, ura3, his3, ade2, can1 (s2)*), modified as follows to create the sensor strains. Yeast sensor strains were made by integrating the Gal4-N-Ada expression cassette (contained in pJAC90/pJAC91) and the EGFP reporter cassette (contained in pJAC92, pJAC93, pJAC98, and pJAC100) into the *his3* and *trp1* loci, respectively. Table S3 summarizes the genotypes of the yeast strains used in this work. Yeast sensor strains were grown on standard dextrose (SD) complete media (Difco) for transfer function and soil detection assays. Yeast strains were grown in SD-Ura media for MHT experiments to maintain the MHT plasmids.

Plasmid Construction

All plasmids were constructed using the Chew-Back, Anneal, and Repair (Gibson) method[79]. The *E. coli* Ada sensor reporter plasmid pFM45 was derived from pSB3K3[80,81] and contained the native *ada* promoter (−80 to +1) driving GFPmut3b fluorescent protein[82], a p15A origin of replication, and a kanamycin resistance marker. This plasmid is comparable to the standard promoter reference plasmid created by Kelly, et al. (2009)[83]. Plasmid pFM141 contains *ada* downstream of the P$_{BAD}$ promoter and medium strength RBS (B0032) as well as the *araC* gene, a spectinomycin resistance marker, and the incW origin of replication. Plasmids pJAC90 and pJAC91 were derived from the shuttle vector pNH603 (derived from pRS303, Addgene) and contained promoter P$_{Adh1}$ and P$_{Cyc1}$, respectively, driving Gal4-N-Ada fusion expression as well as *his3* homology regions flanking the Gal4-N-Ada expression cassette, the *E. coli* colE1 origin of replication, and an ampicillin resistance marker. The Gal4-N-Ada sequence in pJAC90/pJAC91 is a fusion of the Gal4 activation domain (amino acids 768–881), an intervening GSGSGSGS linker, and the N-terminal domain of Ada (amino acids 1–180). Yeast sensor reporter cassette plasmids pJAC100, pJAC92, pJAC93, and pJAC98 were derived from the pNH604 vector and contained 0, 1, 3, and 8 Ada operator sequences (A<u>AAT</u>TAAAGC<u>G</u>CAA; consensus underlined)[84], respectively, upstream of a P$_{Cyc1}$ promoter driving yeast-optimized enhanced green fluorescent protein (EGFP)[85]. Ada operator repeats were generated by iteratively cutting and ligating two annealed, 5'-phosphorylated oligos (5'-GGCCCGAAAAATTAAAGCGCAAGATGC-3' and 5'-GGCCGCATCTTGCGCTTTAATTTTTCG-3') into pJAC92 with enzyme PspOMI. The PspOMI site is fully re-constituted on the 5' end of the double stranded oligo, but broken on the 3' end such that iterative insertion of the oligo then re-digestion with PspOMI allows expansion of the number of operators. Plasmids pJAC90/pJAC91 and pJAC92/pJAC93/pJAC98/pJAC100 were transformed into *S. cerevisiae* SO992 using a standard lithium acetate technique and their flanked expression cassettes were integrated into the *his3* and *trp1* loci, respectively[86]. All plasmids, their components, and GenBank accession numbers are listed and described in detail in the Supporting Information.

Preparation of alkylating agents

Alkylating agents used for induction included methyl iodide (MeI; Sigma-Aldrich #289566), methyl methanesulfonate (MMS; Aldrich #129925), ethyl methanesulfonate (EMS; Sigma M0880), dimethyl sulfate (DMS; Sigma-Aldrich #D186309), and 1-methyl-3-nitro-1-nitrosoguanidine (MNNG; Aldrich #129941). MNNG was dissolved in DMSO. When sensor cultures were exposed to DMSO alone, no induction of fluorescence was observed (data not shown). To make accurate dilutions of MeI, it was important to first make a 1:100 water dilution of pure MeI in 1.5 ml Eppendorf tubes and then vortex the solution several times over five minutes to thoroughly dissolve the MeI before adding it to the 96-well plate. Higher concentrations of MeI required direct addition of MeI to the cultures, which must be done quickly and carefully given the compound's volatility.

E. coli response function assays

These assays were performed in 96-well plates (PlateOne #1896–2000). Triplicate cultures of *E. coli* MG1655 carrying plasmids were grown overnight (~18 hrs) in 3 ml of supplemented M9 media plus antibiotics and were diluted back 1:100 into 1 ml of media into the wells of the 96-well plate. The plate was covered with a breathable membrane (USA Scientific #9123–6100). Cultures were grown for 3 hours at 37°C while shaken at 900 RPM in a plate incubator in a fume hood until early exponential phase ($OD_{600} = 0.2$) and were then induced. For induction, 50× solutions of alkylating agents were first prepared in wells of a 96-well plate, so that a 12-channel pipette could be used to pipette 20 μl of the 50× solution in parallel into the rows of the 96-well culture plate. After alkylating agents were added, the 96-well plate was covered with an airtight sealing mat (Genesee Scientific #22–517) to prevent excessive evaporation of the alkylating agents. Once induced, cells were grown for 3 hours as described above. Cells were collected by pipetting 2 μl of each culture into 200 μl of cold phosphate buffered saline (PBS; pH 7) and 2 mg/ml kanamycin (to stop translation) in a 96-well cytometry plate (Costar #3363). These samples were then analyzed by cytometry as described.

S. cerevisiae response function assays

S. cerevisiae transfer function assays were carried out similarly to *E. coli* assays in 1 ml cultures in 96-well plates. Triplicate cultures of *S. cerevisiae* were grown overnight in Standard Dextrose (SD) media on a rotator (New Brunswick TC7) at 80 RPM at 30°C. The next day, cultures were diluted back 1/100 in SD, and grown to OD_{600} of 0.04 on a shaker (Eppendorf MixMate) at 800 RPM. Methylating agents were added to the cultures as described above and growth was continued for an additional 3 hours. Cells were collected by adding 10 μl of culture to 200 μl of cold PBS and 5 μg/ml cyclohexamide (Sigma C1988) to arrest translation in 96-well plates. These samples were then analyzed by cytometry as described.

Cytometry and data analysis

Cells were analyzed by flow cytometry on a BD LSRII using a 488 nm laser and 510/20 nm band pass filter to collect GFP and EGFP fluorescence. Samples of up to 40 μl of cells in cold PBS were analyzed at a flow rate of 0.5 μl/s until 50,000 gated counts were collected. FSC-H and SSC-H thresholds were set to exclude background events. For accurate, reproducible fluorescence measurements, it was critical that cells were diluted at least 100-fold ($OD_{600}<0.04$) so the event rate was low enough for individual cells to be measured. Data was analyzed using FlowJo software (Treestar). The cell populations were gated by time and forward/side scatter to exclude read-through from previous wells and residual background events. The final analyzed populations included >90% of collected events. The geometric mean of the fluorescence histogram of each gated population was calculated and

is reported here as the fluorescence value of a sample in arbitrary units (au). Modeling and fitting of response functions was done in Matlab using the *nlinfit* function applied to the model presented in the Supporting Information. Fit data sets excluded data points where cells experienced toxicity.

Detection of MeI production by MHTs

The *S. cerevisiae* $P_{8x.Cyc1}|P_{Adh1}$ sensor strain was transformed with the methyl halide transferase (MHT) expression plasmids previously described[37]. Plasmids expressing MHT's from the following organisms were tested: *Batis maritima, Burkholderia pseudomallei, Burkholderia xenovorans, Vitis vinifera, Burkholderia thailandensis, Brassica rapa*, and *Oryza sativa*. Transformants were grown overnight in 2 ml SD-Ura selective media to retain the MHT plasmids. The following day, cultures were added to 100 ml of fresh SD-Ura and grown for 24 hours. Cultures were centrifuged to pellet the cells, then resuspended in 8 ml YPD (final $OD_{600} = 50$) and 1 ml of 1 M NaI as a source of iodide. Cells were grown for 1 hour in 14 ml Falcon tubes (Becton Dickinson #35209) sealed with Septa Seal rubber stoppers (Sigma #124605). Gas chromatography-mass spectrometry (GC-MS) was conducted using a model 6850 Series II Network GC system and model 5973 Network mass-selective system (Agilent). GC-MS measurements were done as previously described[37], except for the following changes: the oven temperature was set at 55°C and increased to 70°C over a period of 9 minutes so as to process all samples, including the standard curve, in one run. Samples were injected 30s apart so that their MeI GC peaks were clearly separated and identifiable with respect to the air peak. A sample of the remaining cells was diluted 1:1000 in SD media and grown for an additional 3 hours before being assayed for fluorescence by cytometry as described. To better illustrate how fluorescence changed with MeI production, the fluorescence background of the $P_{8x.Cyc1}|P_{Adh1}$ sensor strain was subtracted from the fluorescence measurements of each of the experimental strains measured. This was only done for the data in Figure 3A; all other data does not have the background subtracted.

Detection of MeI contamination in soil samples

Garden soil was added to the 1 ml fill line (~230 mg) in a 1.5 ml Eppendorf tube. Then, 800 µl of distilled water was added to the soil and the sample was briefly vortexed. To half the samples, MeI was added to a final concentration of 0.6 mM with respect to the water, simulating the amount added to the soil in agriculture[59]. No MeI was added to control samples. After addition of water and MeI, all samples were vortexed for 20 s and then placed in a dark fume hood at room temperature (20°C). Half the sample tubes were left closed and the other half open. Samples containing MeI and control (water only) soil samples were set up 0, 1, 2, 4, 8, and 24 hours prior to processing. Processing was done as follows: all tubes were closed, the tubes were tapped to bring the soil sample to the top, and a hot 26 gauge needle was used to pierce the bottom of the sample tubes. Pierced tubes were then placed in 1.5 ml Eppendorf collection tubes and centrifuged at 10,000 RPM for 15 s. This served to separate the majority of the liquid fraction from the soil. Samples were then centrifuged further in closed caps for 5 min at 13,500 RPM, after which 400 µl of liquid was removed, taking care to avoid picking up solid material with the pipette. This supernatant was then diluted 1:10 in water and 40 µl of this dilution was added to 1 ml cultures of *S. cerevisiae* $P_{8x.Cyc1}|P_{Adh1}$ reporter cells in a 96-well plate in triplicate. The cells were shaken at 800 RPM at 30°C for 3 hrs and then measured by flow cytometry as described.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Mechanism and activity of *E. coli* and *S. cerevisiae* methylation sensors
(**A**) Diagram of the *E. coli* methylation sensor. The Ada protein is expressed from either the genome or a low-copy plasmid (inset in lower right). (**B**) Overlaid histograms show the induction of *E. coli* MG1655 carrying the plasmid pFM45. The line colors correspond to different concentrations of MeI: 0 (light blue), 98 μM (dark blue), 244 μM (violet), 610 μM (pink), and 3.8 mM (red). (**C**) Response functions for the methylation sensor are shown in *E. coli* MG1655. Solid lines are strains expressing inducible Ada from plasmid pFM141 with variable amounts of arabinose: 0 mM (circles), 1 mM (triangles), and 10 mM (diamonds). *E. coli* containing only the reporter plasmid pFM45 is shown for comparison (black squares,

dashed line). **(D)** Response functions for the methylation sensor are shown for strain *E. coli* MG1655Δ*ada*. The same symbols and arabinose concentrations are used as in C, above. **(E)** Diagram of the *S. cerevisiae* methylation sensor. The sensor protein (Gal4-N-Ada) is expressed from the genome (inset in lower right). **(F)** The histogram shows the induction of *S. cerevisiae* carrying the $P_{8x.Cyc1}|P_{Adh1}$ methylation sensor. The line colors correspond to different concentrations of MeI: 0 (light blue), 148 uM (dark blue), 783 uM (violet), 9.5 mM (red). **(G)** Response functions of the methylation sensor in *S. cerevisiae* with variable number of operators upstream of the reporter promoter. Yeast sensor strains with promoter $P_{0x.Cyc1}$ (circles), $P_{1x.Cyc1}$ (triangles), $P_{3x.Cyc1}$ (diamonds), and $P_{8x.Cyc1}$ (squares) contain corresponding numbers of Ada operators upstream of reporter promoter P_{Cyc1}. The inset shows which symbols correspond to the number of operators in the promoter. The Gal4-N-Ada sensor protein in these strains is driven by the P_{Adh1} promoter. **(H)** Response functions of the methylation sensor in *S. cerevisiae* with Gal4-N-Ada expression driven by either the strong promoter P_{Adh1} (dark blue squares) or the weaker promoter P_{Cyc1} (light green squares). Both strains contain the EGFP reporter driven by promoter $P_{8x.Cyc1}$. Diagonal grey dashes indicate MeI concentrations observed to be toxic (Figure S4). The $P_{8x.Cyc1}$ (squares) data is the same in both **(G)** and **(H)** for ease of comparison. Lines were generated by the sensor activation model described in the Supporting Information and are matched to their respective data by color. For all data, error bars represent one standard deviation from three independent experiments performed on different days.
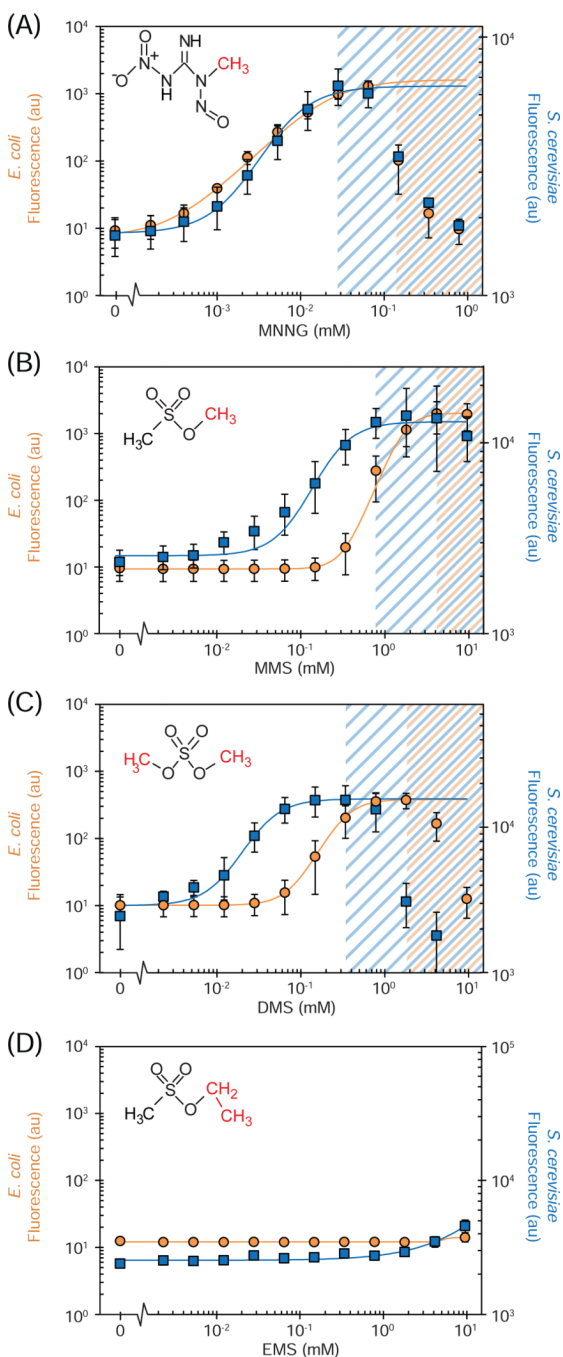
Figure 2. Response of the methylation sensors to S_N1 and S_N2 alkylating agents
E. coli MG1655 carrying plasmid pFM45 and *S. cerevisiae* sensor $P_{8x.Cyc1}|P_{Adh1}$ were
exposed to: **(A)** methylnitronitrosoguanidine (MNNG), **(B)** methyl methanesulfonate
(MMS), **(C)** dimethyl sulfate (DMS), and **(D)** ethyl methanesulfonate (EMS). *E. coli*
(orange circles) and *S. cerevisiae* (blue squares) data correspond to the left and right axes,
respectively. Insets are the structures of the respective alkylating agents, with the donated
alkyl group highlighted in red. Blue and orange shaded regions indicate concentrations of
alkylating agent higher than the LD_{50} for *S. cerevisiae* and *E. coli*, respectively. The *S.
cerevisiae* axis is scaled so that the highest and lowest values of the *E. coli* and *S. cerevisiae*

curves are aligned for easier comparison. Toxicity of EMS was not measured. Error bars are one standard deviation from three independent experiments performed on different days.
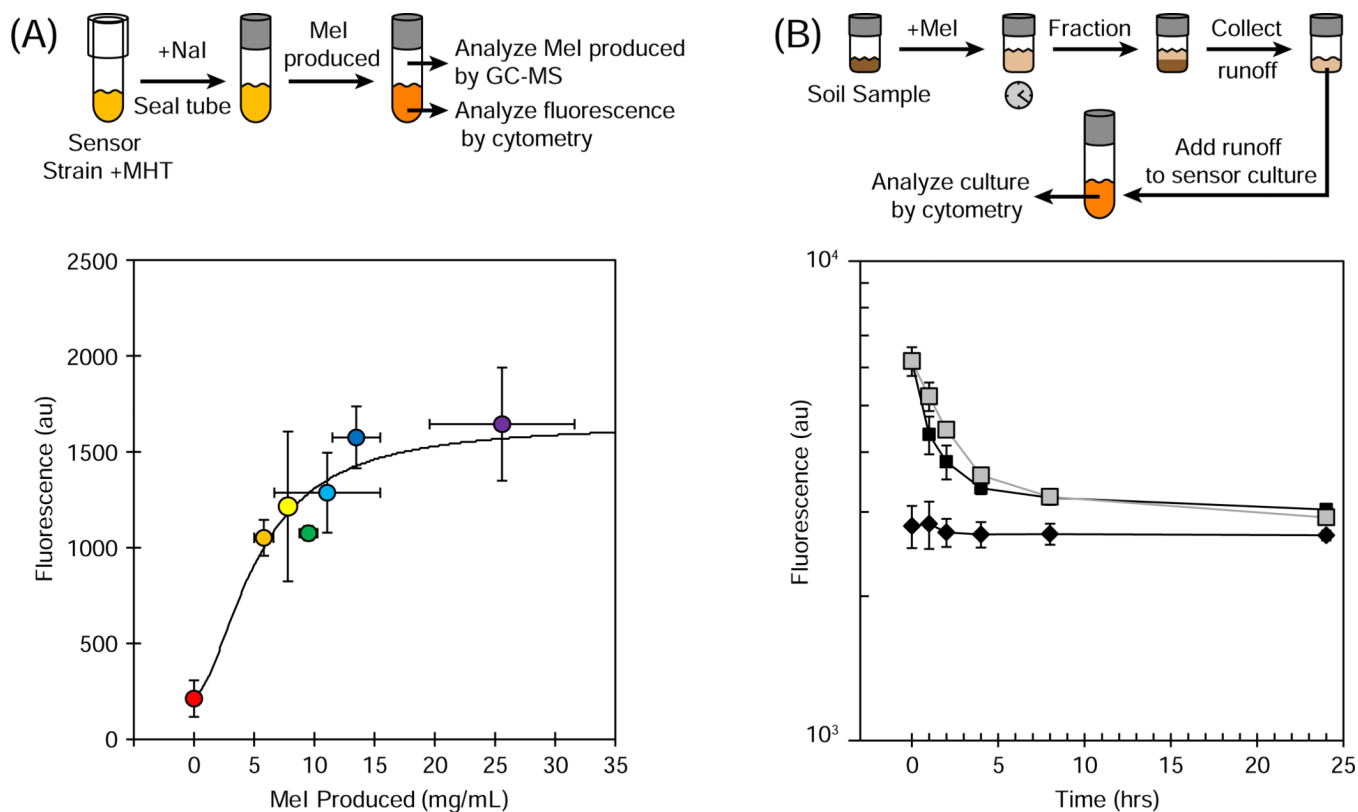
Figure 3. The yeast sensor detects MeI in MeI-producing cultures and MeI-contaminated soil
(A) The $P_{8x.Cyc1}|P_{Adh1}$ sensor was used to screen a collection of methyl halide transferase (MHT) enzymes expressed from a plasmid co-transformed into *S. cerevisiae*. The MeI produced by each MHT as measured by GC-MS correlates with the fluorescence output of the sensor. The solid line shows a fit to a saturating function (Supporting Information). MHT homologues were derived from: *Batis maritima* (violet), *Burkholderia pseudomallei* (dark blue), *Burkholderia xenovorans* (light blue), *Vitis vinifera* (green), *Burkholderia thalandensis* (yellow), *Brassica rapa* (orange), and *Oryza sativa* (red). Error bars are 1 standard deviation from three experiments performed on different days. **(B)** The experimental design for testing sensor activity in soil samples and the resulting data are shown. Tubes to which MeI was added at time 0 are shown as squares. Light squares represent tubes that were closed at time 0 and dark squares represent tubes that were left open to assess the impact of evaporation. Tubes to which only water was added to the soil are shown as black diamonds.

Table 1

Performance of *E. coli* methylation sensors in response to MeI.

Organism	Sensor	Arabinose (mM)	Detection Threshold (μM)	Sensitivity[e] (au/ μM)	Basal Activity[f]	Dynamic Range (fold-change)	Cooperativity[g]
E. coli	a,b	0	100	1.3 ±0.4	0.97 ±0.01	260 ±86	2.3 ±0.6
	a,c	0	40	1.9 ±0.8	1.0 ±0.03	350 ±39	3.2 ±0.7
	a,c	1	16	1.9 ±0.8	1.6 ±0.2	230 ±34	2.4 ±0.2
	a,c	10	6	2.3 ±0.7	16 ±6.7	49 ±18	1.5 ±0.6
	a,c,d	0	40	0.2 ±0.04	1.2 ±0.2	51 ±14	1.5 ±0.4
	a,c,d	1	16	0.8 ±0.2	1.5 ±0.2	155 ±81	1.3 ±0.7
	a,c,d	10	6	2.0 ±0.2	13 ±2.5	40 ±8	1.3 ±0.6

[a.] *E. coli* MG1655 containing pFM45

[b.] *E. coli* MG1655 expressing sensor protein from native locus on genome

[c.] *E. coli* MG1655 expressing sensor protein from PBad promoter on pFM141

[d.] *E. coli* MG1655 containing the Δ*ada* mutation

[e.] Sensitivity is the difference between the minimum and maximum outputs divided by difference in inducer concentration at the detection threshold and the maximum output.

[f.] Basal activity is reported in multiples of the background fluorescence of cells containing no reporter GFP.

[g.] Cooperativity is reported as the Hill coefficient in the fit equations detailed in the SI.

Table 2

Performance of *S. cerevisiae* methylation sensors in response to MeI.

Organism	Sensor	#Operators[c]	Detection Threshold (μM)	Sensitivity[d] (au/μM)	Basal Activity[e]	Dynamic Range (fold-change)	Cooperativity[f]
S. cerevisiae (SO992)	*a*	1	28	0.22 ±0.01	2.9 ±0.05	3.1 ±0.1	1.4 ±0.4
	a	3	28	0.33 ±0.09	2.4 ±0.04	4.9 ±1.1	1.5 ±0.8
	a	8	28	0.42 ±0.03	2.8 ±0.10	5.2 ±0.4	1.6 ±0.3
	b	8	340	0.35 ±0.02	2.7 ±0.13	4.4 ±0.3	1.8 ±0.9

[a]·Contains PAdh1 driving the expression of Gal4-N-Ada

[b]·Contains PCyc1 driving the expression of Gal4-N-Ada

[c]·Operators in the PCyc1 promoter driving EGFP

[d]·Sensitivity is the difference between the minimum and maximum outputs divided by difference in inducer concentration at the detection threshold and the maximum output.

[e]·Basal activity is reported in multiples of the background fluorescence of cells containing no reporter GFP.

[f]·Cooperativity is reported as the Hill coefficient in the fit equations detailed in the SI.

Table 3

Response of the methylation sensors to different agents.

Organism	Detection Threshold (μM)			Dynamic range (fold-change)			Cooperativity[c]		
	MMS	DMS	MNNG	MMS	DMS	MNNG	MMS	DMS	MNNG
E. coli [a]	783	340	1	222 ±45	41 ±14	171 ±39	3.0 ±0.7	2.7 ±0.3	1.3 ±0.2
S. cerevisiae[b]	28	2	2	6.4 ±1.9	6.9 ±2.0	3.8 ±0.2	1.4 ±0.4	2.0 ±0.7	1.4 ±0.1

[a] E. coli MG1655 containing pFM45

[b] S. cerevisiae containing P8x.Cyc1 and PAdh1 driving the expression of Gal4-N-Ada

[c] Cooperativity is reported as the Hill coefficient in the fit equations detailed in the SI.