Novel Biochemical Properties and Physiological Role of the Flavin Mononucleotide Oxidoreductase YhdA from *Bacillus subtilis*

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ABSTRACT Cr(VI) is mutagenic and teratogenic and considered an environmental pollutant of increasing concern. The use of microbial enzymes that convert this ion into its less toxic reduced insoluble form, Cr(III), represents a valuable bioremediation strategy. In this study, we examined the *Bacillus subtilis* YhdA enzyme, which belongs to the family of NADPH-dependent flavin mononucleotide oxide reductases and possesses azo-reductase activity as a factor that upon overexpression confers protection on *B. subtilis* from the cytotoxic effects promoted by Cr(VI) and counteracts the mutagenic effects of the reactive oxygen species (ROS)-promoted lesion 8-OxoG. Further, our *in vitro* assays unveiled catalytic and biochemical properties of biotechnological relevance in YhdA; a pure recombinant His10-YhdA protein efficiently catalyzed the reduction of Cr(VI) employing NADPH as a cofactor. The activity of the pure oxidoreductase YhdA was optimal at 30°C and at pH 7.5 and displayed $K_m$ and $V_{max}$ values of 7.26 mM and 26.8 μmol·min$^{-1}$·mg$^{-1}$ for Cr(VI), respectively. Therefore, YhdA can be used for efficient bioremediation of Cr(VI) and counteracts the cytotoxic and genotoxic effects of oxygen radicals induced by intracellular factors and those generated during reduction of hexavalent chromium.

IMPORTANCE Here, we report that the bacterial flavin mononucleotide/NADPH-dependent oxidoreductase YhdA, widely distributed among Gram-positive bacilli, conferred protection to cells from the cytotoxic effects of Cr(VI) and prevented the hypermutagenesis exhibited by a MutT/MutM/MutY-deficient strain. Additionally, a purified recombinant His$_{10}$-YhdA protein displayed a strong NADPH-dependent chromate reductase activity. Therefore, we postulate that in bacterial cells, YhdA counteracts the cytotoxic and genotoxic effects of intracellular and extracellular inducers of oxygen radicals, including those caused by hexavalent chromium.

KEYWORDS *Bacillus subtilis*, bioremediation, chromate, mutagenesis, oxidoreductases, oxygen radicals

Anthropogenic activity has converted chromium (Cr), an element found in rocks, soils, plants, and animals, into a dangerous environmental pollutant. The water-soluble, hexavalent species Cr(VI) permeates into the cell, presumably through the sulfate transporter systems (1, 2), interacts with intracellular redox compounds, can be partially reduced to Cr(IV), and generates reactive oxygen species (ROS) that damage distinct biomolecules (3–7). ROS can attack DNA and produce a multiplicity of genetic lesions, including 8-OxoG, apurinic/apyrimidinic sites (AP), and single- and double-strand breaks (8–10). Results from *in vitro* and *in vivo* studies showed that the presence of Cr(VI) induces a broader repertoire of genetic insults like DNA-protein cross-links...
Evolution has equipped microorganisms with several strategies to contend with the cytotoxic and genotoxic effects of Cr(VI), including efflux of chromate ions from the cell’s cytoplasm, incision/excision repair of ROS-promoted DNA damage, and enzymatic reduction of the hexavalent ion (14–16). Bioremediation of Cr(VI) with chromate reductases is considered an efficient alternative to chemical cleanup strategies (17, 18). Bacterial conversion of Cr(VI) to Cr(III) can proceed under aerobic conditions with reductases that use NADH or NADPH as a cofactor or under anaerobiosis employing enzymes that use hexavalent chromate as an electron acceptor (19, 20).

While several microbial enzymes can catalyze the transfer of one electron and thus reduce Cr(VI) to Cr(V), such reductases are not of biotechnological value because Cr(V) elicits the production of oxygen radicals that are toxic to cells (6, 7). Therefore, microbial remediation with oxidoreductases that possess the ability to reduce Cr(VI) to Cr(III), avoiding the intermediates Cr(V) and Cr(IV), are of significant biotechnological value.

Two enzymes (EC 1.6.5.2) that fit these properties are ChrR from Pseudomonas putida (PpuChrR) and YieF from Escherichia coli (EcoYieF), which have been studied extensively (15, 21–23). ChrR is a soluble and flavin mononucleotide (FMN)-binding protein dimer capable of transiently generating flavin semiquinone during chromate reduction with transfer of electrons from NADH to superoxide anions. However, production of the Cr(V) intermediate and the superoxide anion is transient, which protects P. putida from chromate toxicity by avoiding the cellular one-electron reduction and minimizing the generation of ROS (15). In contrast, the E. coli oxidoreductase YieF reduces chromate through a four-electrons transfer mechanism: 3 electrons to hexavalent chromium to produce Cr(III) and 1 electron to molecular oxygen, which result in minimal production of ROS (21). The structural and functional aspects of ChrR and YieF during chromate reduction have been reported previously (23); however, less is known about the physiological role of these proteins in the cell. Interestingly, the biological function of YieF remains unclear (21, 23), but ChrR reduces quinones and protects P. putida from hydrogen peroxide (15).

Here, we report that YhdA, a protein from B. subtilis (BsuYhdA), showed azoreductase activity (24, 25) and amino acid similarity with the NAD(P)H FMN-dependent chromate reductases ChrR and YieF, respectively. Overexpression of yhdA conferred protection on B. subtilis from the cytotoxic effects of Cr(VI) and prevented the hypermutagenesis exhibited by a MutT/MutM/MutY-deficient B. subtilis strain. Additionally, a purified recombinant His_{10}-YhdA protein displayed a strong NADP(H)-dependent chromate reductase activity. Therefore, we postulate that in B. subtilis, YhdA counteracts the cytotoxic and genotoxic effects of oxygen radicals, including those generated during the reduction of Cr(VI).

RESULTS

YhdA possesses structural homology with chromate reductases. Previous observations revealed that Cr(VI) induced an adaptive response in B. subtilis and that vegetative cells of this microorganism exhibited the capacity to reduce this toxic hexavalent ion (13). To improve our view of this response, the genome of B. subtilis was inspected for genes encoding proteins with amino acid homology to the two bona fide chromate reductases YieF and ChrR from E. coli and P. putida, respectively (21). Results from a CLUSTALW multiple-sequence analysis (Fig. 1) revealed that yhdA possessed 53% and 58% amino acid similarity with the chromate reductases PpuChrR and EcoYieF, respectively. As shown in Fig. 1, a higher level of amino acid similarity was detected among the three proteins in the signature sequence LFVTPEYNXXXXXXLKNAIDXXS, which is widely conserved in the NADH-dh2 protein family, consisting of bacterial and eukaryotic NAD(P)H oxidoreductases (24–26). Additionally, YhdA uses NADPH as the electron donor to reduce the active-site FMN, which is also necessary for chromate reduction in ChrR and YieF (21). Analysis of the crystal structure of YieF complexed with FMN revealed that this cofactor is anchored by hydrogen bonds to a strand-loop-helix nucleotide-binding motif, GSLRKGSFN, located on loop 1 of the protein (23); of note, several conserved residues of this motif are present in YhdA (PDB code 1NNI) (27).
A careful comparison among the crystal structures of BsuYhdA and EcoYieF (PDB code 3SVL) revealed that only three amino acids (Gly8, Arg11, and Lys12) are perfectly conserved in this motif. Additional amino acids implicated in FMN complexation in YieF include Glu82, Tyr83, Asn84, Tyr85, and Ser117 (23). As shown in Fig. 1B, equivalent residues in YhdA, including Glu73, Tyr74, His75, and Ser76, are located at positions similar to those in EcoYieF (Fig. 1B). Interestingly, YhdA contains an Asn residue in position 116 instead of a His residue in PpuChrR or Tyr in EcoYieF (Fig. 1A). Results derived from a previous study indicated that substitution of this residue for Asn greatly improves the chromate reductase activity in EcoYieF (23). Based on this structural analysis, we hypothesized that YhdA, in addition to its azoreductase activity (24), can catalyze the reduction of Cr(VI).

**YhdA confers protection on B. subtilis from the noxious effects of chromate.** As noted above, YhdA shares structural homology with the chromate reductases PpuChrR and EcoYieF, and results from a previous study revealed that Cr(VI) impacted *B. subtilis* viability through a mechanism of ROS-promoted cell damage (13). Therefore, we first inquired whether YhdA, by acting as a chromate reductase, confers *in vivo* protection on *B. subtilis* cells from Cr(VI) toxicity. This aspect was initially addressed employing a *B. subtilis* strain bearing a disrupted yhdA gene (Table 1). Our results revealed no
significant differences in the levels of resistance to the noxious effects of Cr(VI) between the YhdA-deficient mutant and its parental proficient strain (Fig. 2). Thus, the YhdA-deficient and wild-type parental strains exhibited 90% lethal dose values (LD$_{90}$) of 58.5 ± 2.8 and 60 ± 2.5 mg liter$^{-1}$ of Cr(VI), respectively (Fig. 2). Considering these results, we designed the vector pDG148 to overexpress yhdA under the control of the isopropyl-$eta$-D-thiogalactopyranoside (IPTG)-inducible promoter $P_{spac}$ (Table 1). A B. subtilis strain carrying this construct produced a protein with the molecular mass expected for YhdA (i.e., ∼20 kDa) in a time-dependent manner and when the lactose analogue IPTG was present in the medium (Fig. 3A). Results from Fig. 3B revealed that the cell extracts of a control strain carrying the empty vector pDG148 displayed significantly lower levels of chromate reductase activity in cell extracts of the control strain, such values increased even more in the strain that overexpressed yhdA (these increases were statistically significant at a $P$ value of <0.05 [Fig. 3B]).

We then treated the strain that overproduces YhdA or that carrying the empty vector pDG148 with increasing doses of chromate, and the fraction of survivors was determined by viable counts. The results showed that the strain overexpressing yhdA significantly increased its resistance to Cr(VI) exposure (Fig. 4). The yhdA overexpressing and control strains exhibited LD$_{90}$ values of 220 ± 15 mg ml$^{-1}$ and 72 ± 7 mg ml$^{-1}$ of Cr(VI), respectively (Fig. 4B). Together, these results strongly suggest that YhdA reduces hexavalent chromium and protects B. subtilis during exposure to this pollutant.

### Table 1: Plasmids and strains employed in this study

<table>
<thead>
<tr>
<th>Plasmid or strain</th>
<th>Genotype or description$^a$</th>
<th>Source or reference$^b$</th>
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<tr>
<td>pDG148</td>
<td>Shuttle IPTG-inducible $P_{spac}$ vector; Amp$^\beta$ Kan$^\beta$</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>pET19b</td>
<td>Expression vector containing $T_\beta$ promoter that enables 10×His-tagged protein; Amp$^\beta$</td>
<td>Novagen</td>
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<tr>
<td>pPERM1556</td>
<td>pET19b containing a 519-bp fragment from the $yhdA$ ORF lacking the start and stop codons and cloned between the NdeI/BamHI restriction sites; Amp$^\beta$</td>
<td>This study</td>
</tr>
<tr>
<td>pPERM1557</td>
<td>pDG148 with a 702-bp fragment encompassing the ORF and extending 177 bp downstream of the stop codon of $yhdA$ and cloned between the SalI/HindIII restriction sites; Kan$^\beta$</td>
<td>This study</td>
</tr>
<tr>
<td>pPERM1803</td>
<td>pMUTIN4-Cat containing a 5’ fragment (from bp 40 to 274) of the $yhdA$ ORF cloned into the HindIII-BamHI sites; Cm$^\beta$</td>
<td>This study</td>
</tr>
</tbody>
</table>

$^a$Selection markers: Amp, ampicillin; Cm, chloramphenicol; Kan, kanamycin; Sp, spectinomycin; Tc, tetracycline.

$^b$X→Y, strain X was transformed with DNA from source Y.
Heterologous synthesis and purification of a His\textsubscript{10}-YhdA protein. To demonstrate that yhdA codes for a protein that catalyze the reduction of hexavalent chromium, we designed a recombinant YhdA protein for purification and \textit{in vitro} assays. To this end, the open reading frame (ORF) of yhdA lacking the first codon was PCR amplified and cloned in the expression vector pET19b and the gene product was expressed from the IPTG-inducible T\textsubscript{7} promoter to generate a His\textsubscript{10}-YhdA protein. The resulting construct (pPERM1556) was first replicated in \textit{E. coli} DH5\textsubscript{a}/H9251 (strain PERM1553) and then transformed into \textit{E. coli} Rosetta, which generated the strain \textit{E. coli} PERM1562. The optimal conditions for yhdA induction in this study were 0.5mM IPTG for 2 to 3 h at 37°C and produced the maximum amount of an ~20 kDa His\textsubscript{10}-YhdA recombinant protein in soluble form (see Fig. S1 in the supplemental material). Cells cultured under these conditions were collected and disrupted as described in Materials and Methods. The His\textsubscript{10}-YhdA protein from the soluble fraction was purified by metal affinity chromatography, and the eluted fractions from this purification protocol contained an ~20-kDa protein with a high level of purity as revealed by SDS-PAGE (Fig. 5A).

\textit{yhdA} encodes a protein with robust chromate reductase activity. As shown in this work, YhdA shares structural similarities with \textit{Eco}YieF and \textit{Ppu}ChrR, previously characterized as FMN oxidoreductases that use NADPH to reduce Cr(VI). Therefore, the His\textsubscript{10}-YhdA pure protein was tested for its ability to catalyze the reduction of 50 ppm of Cr(VI) in the presence of 10 mM NADPH at 30°C. Under these conditions, His\textsubscript{10}-YhdA displayed a specific activity of 371 ± 2.91 mg of Cr(VI) reduced/min/mg of protein. A detailed analysis of the kinetic properties of YhdA, derived from Lineweaver-Burk plots, revealed that His\textsubscript{10}-YhdA reduced Cr(VI) with a \textit{K}_m of 7.26 mM and employed NADPH in the reaction with a \textit{K}_m of 37.3 mM (Fig. 5B and C). Further results showed that His\textsubscript{10}-YhdA was more efficient in reducing hexavalent chromium at 30°C and at a pH of 7.5 (Fig. 6).

Physiological role of YhdA in counteracting the mutagenic effects of 8-OxoG. Chromate kills \textit{B. subtilis} cells by increasing the synthesis of oxygen radicals and the levels of 8-OxoG lesions (13). Importantly, this type of oxidative DNA damage is efficiently processed by the error prevention oxidized guanine system (GO) in this bacterium (13). Based on these observations and the results shown in Fig. 4, we investigated if YhdA prevents the formation of mutations in \textit{B. subtilis}, mainly those that are increased in the absence of systems that repair 8-OxoG lesions. To test this notion, the IPTG-inducible Pspac-yhdA cassette-bearing plasmid and its empty vector derivative pDG148 were independently transformed into the hypermutagenic strain \textit{B. subtilis} ytkD (mutT) mutM mutY (ΔGO) (13). Several reports have demonstrated that this strain
is \~3 orders of magnitude more mutagenic than its GO-proficient counterpart (13, 28). Notably, the overexpression of yhdA resulted in a significant decline in the spontaneous mutagenesis of the ΔGO strain (from 2,130 ± 350 to 135 ± 14 rifampin-resistant [Rifr] mutants in 10⁹ cells) compared to the strain that contained the empty vector, even in the absence of exposure to hydrogen peroxide (Fig. 7). Thus, overexpression of yhdA resulted in 15-fold reduction in Rifr mutagenesis compared to that of the strain with the empty vector. To better assess the antimutagenic properties of YhdA, the ΔGO strains was treated with an LD₅₀ of the ROS promoter agent H₂O₂ and measured for the ability to Rifr mutants. These experiments were conducted in two genetic backgrounds differing in overexpression of yhdA. Oxidant exposure increased mutagenesis levels \~2.5 times compared to the untreated condition (from 2,130 ± 350 to 5,130 ± 430 Rifr mutants in 10⁹ cells) in the strain containing the empty vector. When cells differing in yhdA expression experienced oxidant exposure, Rifr mutagenesis levels decreased \~27 times in the strain that overexpressed yhdA compared to the strain with the empty vector. Together, these results strongly suggest that overproduction of YhdA can reduce the mutagenic effects promoted by endogenously and exogenously induced ROS in B. subtilis.
Results described in this report indicated that the oxidoreductase YhdA from \textit{B. subtilis} possesses structural similarity and biochemical properties displayed by bacterial chromate reductases with biotechnological value. In agreement with these observations, YhdA conferred protection on \textit{B. subtilis} against the deleterious effects of hexavalent chromium; furthermore, a biological role for YhdA in the prevention of mutations promoted by ROS and 8-OxoG lesions was unveiled.

The biotransformation of Cr(VI) to its insoluble and less toxic reduced form Cr(III) by microorganisms is considered an efficient strategy in the treatment of chromium-contaminated waste \cite{12}. As noted above, enzymes like \textit{Ppu}ChrR and \textit{Eco}YieF are appropriate to carry out this process, as they directly catalyze the reduction of Cr(VI) to Cr(III) and limit the intracellular production of toxic oxygen radicals \cite{21}. YhdA most likely shares these valuable properties. In support of this contention, our results showed that increased levels of YhdA significantly improved the capacity of living \textit{B. subtilis} cells to (i) reduce hexavalent chromium, (ii) withstand the toxicity of this pollutant, and (iii) counteract the mutagenic effects of the ROS promoter agent \textit{H}$_2$\textit{O}$_2$. Interestingly, the cell extract of the strain overproducing YhdA reduced Cr(VI) even in the absence of the cofactor NADPH, suggesting that the cells maintain the conditions necessary for the activity of YhdA, which may represent an advantage of this enzyme for bioremediation purposes.

**FIG 4** Effects of hexavalent chromium on the survival of \textit{B. subtilis} cells as affected by overexpression of \textit{yhdA}. Cultures of \textit{B. subtilis} PERM1585 (pDG148) and PERM1568 (pDG148-yhdA) were grown at 37°C in LB medium to an OD$_{600}$ of 0.5. At that point, the strains were supplemented with IPTG (0.5 mM), and after 2 h of induction, they were treated with different concentrations of Cr(VI) for 3 h, all as described in Materials and Methods. The LD$_{90}$ values (B) were calculated for each strain from the dose-response graphs (A). The results show means of data collected from at least three independent experiments performed in triplicate for each sample, and the error bars represent SDs.
FIG 5 Purification (A) and kinetics of chromate reductase reaction (B and C) of recombinant His$_{10}$-YhdA. (A) An LB culture of strain E. coli PERM1562 was grown at 37°C to mid-exponential phase (OD$_{600}$ = 0.5). At that point, the culture was supplemented with IPTG (0.5 mM) and grown for 3 h at 37°C. Cells were pelleted by centrifugation, washed, and disrupted, and the cell extract was subjected to purification by metal affinity chromatography. Aliquots (30 μl) of the purification fractions were analyzed by SDS-PAGE, as described in Materials and Methods. Lane MW, molecular weight markers; lane FT, flowthrough; lanes 1 to 6, protein extracts eluted from the Ni-NTA column with 250 mM imidazole. (B and C) Double-reciprocal plot of chromate reduction by His$_{10}$-YhdA as a function of chromate (B) or NADPH (C) concentration. The apparent $K_m$ and $V_{max}$ values for chromate reduction were estimated by nonlinear regression analysis. Experiments were performed as described in Materials and Methods.
Of note, the ability of *B. subtilis* to proliferate in medium supplemented with chromate resembled what was reported for *P. putida*; the 50% inhibitory concentrations (IC\textsubscript{50}) of the hexavalent ion were \( \approx 400 \) \( \mu \)M and \( \approx 519 \) \( \mu \)M in *P. putida* (21) and *B. subtilis* (Fig. 4), respectively. Furthermore, as shown in this report, the overexpression of *yhdA* increased tolerance to the hexavalent ion by \( \approx 300\% \) in *B. subtilis*. Therefore, together with specific and general DNA repair mechanisms (13, 29), the chromate reductase activity of YhdA plays an important role in the tolerance of *B. subtilis* to Cr(VI).

While several FMN-Red proteins, including YhdA, catalyze the reduction of chromate reductase, several authors attribute a secondary role to this activity (15, 21, 30, 31). Therefore, the physiological role played by these proteins in their hosts has been a matter of concern and remains an open question in many cases, including YhdA. A previous study reported that a *yhdA* knockout strain was able to grow under standard conditions (24). In our study, the susceptibility of a *yhdA*-deficient strain to the noxious effects of Cr(VI) did not significantly differ from that exhibited by its YhdA-proficient parental strain. Therefore, it is possible that *B. subtilis* contends with the cytotoxicity of chromate via different mechanisms, including preventive/repair mechanisms, efflux pumps, and additional FMN oxidoreductases (13, 16, 29) that compensate for the lack of YhdA. However, our approach to overexpress *yhdA* suggested a cellular role for this factor. A biological function of quinone reductase has been attributed to *PpuChrR* and mammalian diaphorase NQO1, among other flavodoxin-dependent reductases (15, 32,

**FIG 6** Optimal temperature (A) and pH (B) for His\textsubscript{10}-YhdA-promoted chromate reduction. Reaction mixtures containing 1 \( \mu \)g of His\textsubscript{10}-YhdA, 10 mM NADPH and 250 ppm Cr(VI) were set to determine the optimal temperature (A) and pH (B) for maximal enzyme activity of His\textsubscript{10}-YhdA, plotted as percentage of maximal activity of Cr(VI) reduction. The values plotted are averages of three independent experiments \( \pm \) SDs.
These proteins catalyze the simultaneous transfer of two electrons to ubiquinone, a component of the electron transport pathway, to produce quinol, which acts as a quencher of ROS and lipid peroxides (34, 35). Flavoenzymes that reduce quinone and catalyze a two-electron transfer reaction are widely distributed in nature (25, 36). For instance, diaphorase (NQO1) protects mammals from the toxicity of ROS-generating carcinogenic compounds like benzo-α-pyrene quinone, naphthoquinone, and benzoquinone (33). We hypothesize that the previously described quinone reductase activity in YhdA (31) protects B. subtilis from free radicals and ROS produced during the one-electron transfer enzymatic reactions (22, 37). In support of this notion, our results revealed that overexpression of yhdA improved the capacity of B. subtilis to proliferate in the presence of high concentrations of Cr(VI), presumably through complete reduction to Cr(III).

Previous reports indicated that yhdA is part of the yhcYZ-yhdA operon, which is under the control of LiaR, a transcriptional regulator of the B. subtilis cell envelope stress response (38). It is possible that the antioxidant capacity of YhdA mitigates the one-electron transfer reactions that produce ROS-generating semiquinones in cellular processes such as those occurring in the electron transport chain. In support of this proposal, the overproduction of YhdA suppressed the hypermutagenic phenotype of a strain that accumulates high levels of the oxidative DNA lesion 8-OxoG. Consequently, our report provides experimental evidence that associates a flavodoxin oxidoreductase activity with a specific mutagenic process in B. subtilis. Of note, in addition to YhdA, B. subtilis produces YocJ (AzoR1) and YvaB (AzoR2), which confer resistance against chemical pollutants like catechol and 2-methylhydroquinone (39). Furthermore, we identified YwqN, a protein of unknown function with amino acid similarity to the chromate reductases YhdA, ChrR, and YieF. Interestingly, YwqN displayed azoreductase activity but did not catalyze the reduction of hexavalent chromium (our unpublished results), which suggests that B. subtilis relies on multiple NADPH-dependent two-electron transfer oxidoreductases to process oxidative stress caused by enzymatic reactions that generate semiquinone intermediates.

A comparison among the crystal structures of BsuYhdA (PDB code 1NNI) and EcoYieF (PDB code 3SVL) revealed that the proteins share similar topologies consisting of an α-β, 3-layer (αβα) sandwich and functional tetrameric quaternary structures. Also, our in silico analysis revealed that YhdA shares additional structural properties with PpuChrR and EcoYieF, including specific motifs to bind the cofactors FMN and NADPH. However,
while YhdA conserved identical residues in these regions, important differences were observed (Fig. 1). Among these differences, YhdA substitutes the conserved residue Tyr128 for Asn116; in EcoYieF, the site-directed substitution of this residue for Asn produced a variant with improved catalytic activity (23). Our in vitro assays with a recombinant His<sub>16</sub>-YhdA protein suggested that the amino acid differences detected in this enzyme possibly affected its catalytic properties. Thus, as shown in Table 2, YhdA exhibited ~30 times lower affinity for hexavalent Cr than PpuChrR and EcoYieF. The former displayed higher <i>k</i><sub>cat</sub> and <i>V</i><sub>max</sub> values than the latter two proteins. Furthermore, as noted above, YhdA was characterized as an enzyme with azoreductase activity (31); notably, the <i>k</i><sub>cat</sub> value for reductive degradation of Cibacron Marine was 0.7 s<sup>-1</sup>, around 10 times slower than the turnover determined for Cr(VI) (namely, 8.93 s<sup>-1</sup>). This result indicates that YhdA is more efficient in reducing chromate than an azo dye. The optimal temperatures and pH ranges reported for chromate reductases range from 30 to 70°C and from 5 to 7.5, respectively (16). Accordingly, YhdA exhibited an optimal temperature of 30°C and a pH of 7.5 during chromate reduction, which are in agreement with those reported for the chromate reductases from <i>Enterobacter cloacae</i> (30°C and pH 7), <i>Vibrio harveyi</i> (30°C and pH 7), and <i>E. coli</i> (37°C and pH 6.5) (16). Furthermore, the optimal temperature for YhdA is similar to the one described for <i>E. coli</i> YieF (35°C) and differs from the one reported for PpuChrR (70°C) (21). In toto, the wide range of substrates, the biochemical parameters, and the antimutagenic properties exhibited by YhdA make this protein a suitable candidate for bioremediation of aquatic environments polluted with Cr(VI) and azo dyes.

### MATERIALS AND METHODS

**Bacterial strains, culture conditions, and reagents.** All <i>B. subtilis</i> and <i>E. coli</i> strains and the plasmids used in this work are listed in Table 1. The growth medium used routinely was lysogeny broth (LB; Lennox formulation). When required, ampicillin (Amp; 100 μg ml<sup>-1</sup>), chloramphenicol (Cm; 5 μg ml<sup>-1</sup>), or kanamycin (Kan; 10 μg ml<sup>-1</sup>) was added to the medium. Liquid cultures were incubated with vigorous aeration (shaking at 250 rpm) at 37°C. Cultures on solid media were grown at 37°C. The optical density (OD) of liquid cultures was monitored with a Pharmacia Ultrospec 2000 spectrophotometer set at 600 nm. A stock solution of potassium dichromate (K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub>) (99.97% purity; JT Baker, Phillipsburg, NJ) at a concentration of 192.32 mM was prepared in sterile Milli-Q water. Working concentrations were prepared by diluting the stock with sterile Milli-Q water and used for the experiments where required.

**Genetic and molecular biology techniques.** Preparation of competent <i>E. coli</i> or <i>B. subtilis</i> cells and their transformation with DNA were performed as previously described (40, 41). Chromosomal DNA from <i>B. subtilis</i> was purified according to a previously described protocol (42). Small-scale preparation of plasmid DNA from <i>E. coli</i> cells, enzymatic manipulations, and agarose gel electrophoresis were performed by standard techniques (41). Medium-scale preparation and purification of plasmid DNA were accomplished by using commercial ion-exchange columns according to the instructions of the supplier (Sigma-Aldrich; St. Louis, MO).

**Generation of a construct to overexpress yhdA.** To generate a <i>B. subtilis</i> strain overexpressing yhdA, a 702-bp fragment encompassing the open reading frame (ORF) of yhdA and extending 177 bp downstream of the stop codon was amplified by PCR using chromosomal DNA from strain <i>B. subtilis</i> 168 and the synthetic oligonucleotide primers 5′-CCAAGCTTATGAACATGTTAGTCATAAATGGC-3′ (forward, containing a HindIII site, underlined) and 5′-GGCTGGACTACAGAGGACATTGGTATC-3′ (reverse, containing a SalI site, underlined). DNA amplification was carried out using Vent DNA polymerase according to the manufacturer’s recommendations (New England BioLabs, Ipswich, MA). The PCR product was digested with HindIII and SalI and cloned into the HindIII/SalI-digested expression vector pDG148 (43). The resulting construct (pPERM1557) was first amplified in <i>E. coli</i> XL-10 Gold and then introduced by transformation into competent cells of <i>B. subtilis</i> IA751 to generate the strain <i>B. subtilis</i> PERM1568 (Table 1). Additionally, a <i>B. subtilis</i> strain carrying the empty vector pDG148 was generated and designated PERM1585 (Table 1).

**Design of an <i>E. coli</i> strain to overproduce and purify a YhdA protein containing a N-terminal His<sub>16</sub> tag.** The ORF of yhdA lacking the first and the stop codons was amplified by PCR, using chromosomal DNA from <i>B. subtilis</i> 168 and the oligonucleotide primer set 5′-GCCATAATGAACATGTTA

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### TABLE 2 Kinetic parameters of <i>Bsu</i>YhdA

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<th>Enzyme</th>
<th>&lt;i&gt;K&lt;sub&gt;m&lt;/sub&gt;&lt;/i&gt; (μM)</th>
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<td>&lt;i&gt;Bsu&lt;/i&gt;YhdA</td>
<td>7.260</td>
<td>8.9</td>
<td>0.119 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>26.8</td>
</tr>
<tr>
<td>PpuChrR&lt;sup&gt;a&lt;/sup&gt;</td>
<td>260</td>
<td>5.8</td>
<td>2.2 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>8.8</td>
</tr>
<tr>
<td>EcoYieF&lt;sup&gt;a&lt;/sup&gt;</td>
<td>200</td>
<td>3.7</td>
<td>1.8 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>5.0</td>
</tr>
</tbody>
</table>

<sup>a</sup>From reference 21.
TABLE 1

<table>
<thead>
<tr>
<th>Chromatographic Conditions</th>
<th>Fractions Eluted</th>
<th>Protein Bound</th>
<th>Protein Eluted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer B alone</td>
<td>200 ml</td>
<td>100 mM Tris-HCl</td>
<td>50 ml</td>
</tr>
<tr>
<td>Buffer B containing 20mM imidazole</td>
<td>600 ml</td>
<td>250mM imidazole</td>
<td>50 ml</td>
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</tbody>
</table>

**Purification of His<sub>10</sub>-YhdA.** E. coli strain PERM1562 was grown at 37°C in 100 ml of LB medium to an optical density at 600 nm (OD<sub>600</sub>) of 0.5. At this point, the culture was supplemented with IPTG to a final concentration of 0.5 mM, and the expression of YhdA was induced for 2 h. Cells were collected by centrifugation and washed twice with 10 ml of 100 mM Tris-HCl (pH 7.5; buffer A) and then disrupted by sonication in 10 ml of buffer A containing lysozyme (2 mg ml<sup>-1</sup>) for 1 h at 4°C. The cell lysate was subjected to centrifugation (27,200 × g) to eliminate undisrupted cells and cell debris, and the supernatant was applied to 5 ml of a nickel-nitrilotriacetic acid (Ni-NTA)-agarose (Qiagen; Valencia, CA) column equilibrated with 100 mM Tris-HCl (pH 7.5)–300 mM NaCl (buffer B). The column was washed with 50 ml of buffer B alone and then with 200 ml of buffer B containing 20 mM imidazole, and then the protein bound to the resin was eluted with 6 ml of buffer B containing 250 mM imidazole. The fractions eluted from the last step were dialyzed overnight in buffer B and stored at −20°C until use. Aliquots (15 µl) were analyzed by SDS-PAGE as previously described (41).

**Generation of a B. subtilis yhdA-deficient strain.** To generate a B. subtilis strain deficient in yhdA, an internal 234-bp fragment from the open reading frame of yhdA was amplified by PCR using chromosomal DNA from strain B. subtilis 168 and the synthetic oligonucleotide primers 5′-GGAGATCCAGCTCCTGGAGATAATCGGCGGACGCAAGCAGAATTGCAGCA-3′ (forward, containing a HindIII site, underlined) and 5′-GGGATCCGGCTGCGGACGGCTGCTTGGAAAATTCCAACGATATTC-3′ (reverse, containing a BamHI site, underlined). DNA amplification was carried out using Vent DNA polymerase according to the manufacturer’s recommendations (New England Biolabs, Ipswich, MA). The PCR product was digested with HindIII and BamHI and cloned into the HindIII/BamHI-cleaved expression vector pMUTIN4-Cat. The resulting construct (pPERM1803) was first amplified in E. coli DH5α and then introduced by transformation into competent cells of B. subtilis IA751 to generate the strain B. subtilis PERM1834 (yhdA::pPERM1803; Cmr) (Table 1).

**Enzyme assays.** Chromate reductase activity was determined by measuring the concentration of residual chromium(VI) remaining in the reactions, after the desired incubation period, and determined spectrophotometrically at 540 nm, using 1,5-diphenylcarbazide (DPC) as the complexing agent, as described previously (44). Cultures of the strains overproducing YhdA (B. subtilis PERM1568) or harboring the empty vector pDG148 (PERM1585) were propagated to reach an OD<sub>600</sub> of 0.5, supplemented with IPTG (0.5 mM), and incubated for 2 h more. The cells from both cultures were pelleted by centrifugation (4000 × g for 15 min at 4°C), washed 2 or 3 times with buffer A (100 mM Tris-HCl, pH 7.5), and subjected to lysisophorization. The dry cells plus 0.2 ml of 0.45- to 0.6-mm-diameter glass beads in 1.5-ml Eppendorf tubes with a small magnetic stirrer were disrupted by 20- to 30-s periods of shaking in a vortex mixer adjusted to the maximum speed. The dry powder was resuspended at 4°C in buffer A supplemented with a protease inhibitor cocktail (Roche, Mannheim, Germany); after elimination of the cell debris by centrifugation, the remaining cell extract was used to determine chromate reductase activity. We set up 0.1-ml reaction mixtures containing 30 µg of total protein in the presence or absence of 10 mM NADPH and 1.923 mM Cr(VI). The reaction mixtures were incubated for 30 min at 30°C and the amount of Cr(VI) remaining was determined as described above. The protein content in the cell extracts was determined with the bicinchoninic acid (BCA) protocol according to the manufacturer’s procedures (Sigma-Aldrich, St. Louis, MO).

The purified recombinant protein His<sub>10</sub>-YhdA was used as a source to calculate the kinetic parameters and the optimal conditions of pH and temperature for reduction of hexavalent Cr. Kinetic studies were conducted with 100 mM Tris-HCl (pH 7.5) at 37°C and 2 to 3 µg of His<sub>10</sub>-YhdA in 0.1-ml reaction mixtures. The apparent K<sub>m</sub> and V<sub>max</sub> values for chromate reduction were estimated by nonlinear regression analysis, under two conditions: (i) with saturating NADPH (10 mM) and various concentrations of Cr(VI) and (ii) with saturating Cr(VI) (4.8 mM) and various concentrations of NADPH. The reaction mixtures were incubated for 10 min, and reduction of hexavalent chromium was determined as described above. Experiments measuring chromate reductase were repeated three times, and values were plotted as averages of duplicate determinations ± standard deviations (SDs).

**Determination of optimal pH and temperature for His<sub>10</sub>-YhdA-dependent Cr(VI) reduction.** To determine the optimum pH, 1 µg of His<sub>10</sub>-YhdA was incubated, at 30°C, with 10 mM NADPH and 4.8 mM Cr(VI) in either 100 mM morpholineethanesulfonic acid (MES; pH 5.5, 6.0, or 6.5) or 100 mM Tris-HCl (pH 7.0, 7.5, 8.5, or 9.5). The optimum temperature was measured in reactions set at 10, 15, 20, 30, 40, or 50°C using mixtures containing 100 mM Tris-HCl (pH 7.5), 1 µg of His<sub>10</sub>-YhdA, 10 mM NADPH, and 4.8 mM Cr(VI). The reaction mixtures were incubated for 30 min and the fraction of hexavalent Cr(VI) remaining was determined as described above. These experiments were repeated three times, and values were plotted as averages of duplicate determinations ± SDs.

**Determination of Cr(VI) toxicity.** To determine the dose-response curves in the survival of B. subtilis cells following Cr(VI) exposure, strain IA751 (PERM110), a ΔyhdA mutant (PERM1834), a strain overexpressing yhdA (PERM1568), and a strain containing the empty vector pDG148 (PERM1585) were grown at 37°C in LB medium to an OD<sub>600</sub> of 0.5. At this stage, cultures of PERM1568 and PERM1585 were supplemented with IPTG (0.5 mM). After an incubation period of 2 h, all the strains were treated with different amounts of chromate for 3 h. Thus, the PERM1568 cultures were supplemented with Cr(VI) concentrations of 0, 0.961, 1.923, 2.884, 3.846, and 5.769 mM and the PERM110, PERM1585, and
PERM1834 cultures were treated with Cr(VI) concentrations of 0, 0.231, 0.462, 0.923, 1.846, and 2.884 mM. After Cr(VI) treatment, bacterial viability was estimated by counting the CFU on LB agar plates. To this end, samples of the bacterial suspensions were collected and serially diluted in 1× phosphate-buffered saline (PBS) (NaCl [137 mM], KCl [2.7 mM], Na2HPO4 [10 mM], and KH2PO4 [1.8 mM]) and aliquots were plated on LB agar plates. Colonies were counted after overnight incubation at 37°C. Data were reported as 90% lethal dose (LD90) values, namely, the concentration of Cr(VI) that kills 90% of the bacterial population.

Determination of spontaneous and H2O2-induced mutation frequencies of B. subtilis ΔGO overexpressing or not overexpressing yhdA. The frequencies of B. subtilis Rif' mutants in the presence or absence of an LD90 of hydrogen peroxide were determined as follows. B. subtilis ΔGO bearing the pDG148 empty vector (PERM1709 [Table 1]) or the pDG148-yhdA construct (PERM1703 [Table 1]) were propagated in LB medium to an OD600 of 1.0; then, each culture was then split into two subcultures. One of the subcultures of each strain was left untreated, and the other was amended with H2O2 to a final concentration of 20 mM. The untreated and H2O2-treated cultures were shaken at 37°C for 16 h. Mutation frequencies were determined with three independent cultures and plating aliquots of each culture, amended with H2O2 or unamended, onto six LB plates containing 10 µg ml⁻¹ of rifampin and 1 mM IPTG. The same procedure was repeated using plates containing no rifampin. The total colonies and Rif' colonies were counted after 24 h of incubation at 37°C.

Statistical analysis. Differences in chromate reductase activity between strains in the absence or presence of NADPH as well as mutagenesis levels between cultures treated with H2O2 or left untreated were calculated by Mann-Whitney U test, and analyses were done using Minitab 17 software. P values of <0.05 were considered significant.

SUPPLEMENTAL MATERIAL
Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

ACKNOWLEDGMENTS
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