A Bacillus Spore-Based Display System for Bioremediation of Atrazine

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ABSTRACT Owing to human activities, a large number of organic chemicals, including petroleum products, industrial solvents, pesticides, herbicides (including atrazine [ATR]), and pharmaceuticals, contaminate soil and aquatic environments. Remediation of these pollutants by conventional approaches is both technically and economically challenging. Bacillus endospores are highly resistant to most physical assaults and are capable of long-term persistence in soil. Spores can be engineered to express, on their surface, important enzymes for bioremediation purposes. We have developed a Bacillus thuringiensis spore platform system that can display a high density of proteins on the spore surface. The spore surface-tethered enzymes exhibit enhanced activity and stability relative to free enzymes in soil and water environments. In this study, we evaluated a B. thuringiensis spore display platform as a bioremediation tool against ATR. The Pseudomonas sp. strain ADP atzA determinant, an ATR chlorohydrolase important to the detoxification of ATR, was expressed as a fusion protein linked to the attachment domain of the BclA spore surface nap layer protein and expressed in B. thuringiensis. Spores from this strain are decorated with AtzA N-terminally linked on the surface of the spores. The recombinant spores were assayed for ATR detoxification in liquid and soil environments, and enzyme kinetics and stability were assessed. We successfully demonstrated the utility of this spore-based enzyme display system to detoxify ATR in water and laboratory soil samples.

IMPORTANCE Atrazine is one of the most widely applied herbicides in the U.S. midwestern states. The long environmental half-life of atrazine has contributed to the contamination of surface water and groundwater by atrazine and its chlorinated metabolites. The toxic properties of ATR have raised public health and ecological concerns. However, remediation of ATR by conventional approaches has proven to be costly and inefficient. We developed a novel B. thuringiensis spore platform system that is capable of long-term persistence in soil and can be engineered to surface express a high density of enzymes useful for bioremediation purposes. The enzymes are stably attached to the surface of the spore exosporium layer. The spore-based system will likely prove useful for remediation of other environmental pollutants as well.

KEYWORDS Bacillus thuringiensis, atrazine, bioremediation, exosporium, spore display

Atrazine (ATR) has been one of the most widely applied herbicides in the U.S. midwestern states. The contamination of surface water and groundwater by ATR and its chlorinated metabolites has raised public health and ecological concerns due to their endocrine disruption activities, potential risk of gastrochisis, and being possibly carcinogenic (1). Remediation of these pollutants by conventional approaches is both technically and economically challenging. The fate of these nitrogen-substituted sym-
metrical triazine ring compounds in the environment is directly correlated with the degradation capability of the soil microbiome. They metabolize these compounds via enzyme-catalyzed hydrolysis reactions (2). Despite the presence of soil organisms with the potential to degrade this herbicide, ATR has a half-life greater than 170 days in soils known to contain ATR-degrading microorganisms (3).

*Pseudomonas* sp. strain ADP was isolated and shown to completely metabolize ATR at concentrations far exceeding the solubility of ATR in water (4). *Pseudomonas* sp. strain ADP can metabolize ATR as its sole nitrogen source during growth, with the ATR ring being cleaved and the carbon atoms liberated as CO₂. The degradation pathway encoded by pADP-1 is shown in Fig. 1. AtzA has been characterized as an ATR chlorohydrolase with restricted substrate specificity that hydrolyzes ATR to hydroxyatrazine (HA) to start the catabolism of ATR to cyanuric acid (5) and further to carbon dioxide and ammonia with the other 5 enzymes encoded in pADP-1 (4–6).

Strain ADP also metabolized ATR under nongrowth conditions, suggesting that immobilized cells might be used for the bioremediation of ATR, which is significant for the potential environmental application of the organism (4). This bacterium can survive and metabolize ATR in a soil environment. However, it requires proper conditions to stimulate the metabolism of ATR, such as the addition of sodium citrate as a suitable carbon source for supporting ATR metabolism (4). This may explain the inefficiency of this organism at metabolizing ATR in soil. An alternative approach to ATR bioremediation involved the treatment of contaminated soil with killed *Escherichia coli* cells engineered to express the AtzA atrazine chlorohydrolase enzyme that can detoxify ATR (7). However, a limitation to this approach is that the enzymatic activity of the killed cells was found to decrease in the chemically complex soil environment. Application of recombinant enzymes directly to soil is a third bioremediation strategy. However, the use of purified recombinant enzymes is expensive, and the enzymes are rapidly inactivated or washed out in soil environments (8).

The targeting and anchoring of heterologous polypeptides or enzymes to the outer surface of bacteriophages, spores, and cells has been a common technique of fundamental and applied research in biotechnology. Spores are particularly well suited for soil bioremediation applications because spores have size, charge, and hydrophobic properties which are ideal for persistence in soil environments (9, 10). Anchoring enzymes to surfaces, such as of spores, has the added advantage of leading to increased activity and stability of the proteins (11). The genus *Bacillus* consists of Gram-positive endospore-forming species that can shift to an alternative developmental pathway, sporulation, when growth conditions become unfavorable (10). Because of the well-characterized genetic systems and lack of pathogenicity of *Bacillus subtilis*, its

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**FIG 1** Catabolic degradation of atrazine by *Pseudomonas* sp. strain ADP. A cluster of enzymes, AtzA, AtzB, AtzC, AtzD, AtzE, and AtzF, in the degradation pathway are encoded in pADP-1 from *Pseudomonas* sp. strain ADP (4). Adapted from reference 5.
spores have been exploited for surface display of recombinant proteins (reviewed in references 12–15). Proteins are displayed on the spore surface through fusions with spore coat proteins. The cotA, cotB, cotC, cotE, cotG, cotX, oxdD, and spcC determinants have been exploited as fusion targets for spore surface display of proteins. Spore display has a number of advantages over other microbial display systems, including ease of production, not requiring the recombinant protein to traverse a membrane, and the inherent stability of spores. A number of reports describe the successful application of B. subtilis spore display systems. The disadvantage of the B. subtilis spore display systems is that spore coat proteins assemble into highly ordered structures and the recombinant fusion proteins may not compete well with the wild-type coat protein for incorporation. Thus, the level of protein incorporation and the degree of surface exposure are variable and dependent on the properties of the foreign protein and the B. subtilis spore protein component of the fusion.

Spores of the Bacillus cereus family (B. anthracis, B. cereus, and B. thuringiensis) possess an outermost spore layer, the exosporium, that is a loosely fitting shell external to the spore coat layer and which is not present on B. subtilis spores (10). The exosporium is comprised of a basal layer and a filamentous hairlike nap layer. Filaments of the hairlike nap are predominantly formed by the collagen-like glycoprotein BclA (16, 17). The BclA glycoprotein is present at a high density over the entire surface of the spores. We identified sequences at the N terminus of the BclA protein that are sufficient for incorporation of the BclA protein onto the exosporium surface and can serve as a fusion point to display foreign proteins on the spore surface (18). The same motif exists in the bclA homolog of B. thuringiensis. We hypothesized that this exosporium surface localization domain would permit spore display of recombinant proteins in the non-mammalian pathogen B. thuringiensis. With the BclA spore surface-targeting domain, it should be possible to display a high density of foreign proteins on the spore surface. In this study, the BclA-based spore display system is demonstrated with B. thuringiensis, and we evaluated the capacity of this spore system to detoxify ATR (19) either in the water or in soil with AtzA-decorated spores.

RESULTS

The B. thuringiensis spore display system. The B. thuringiensis bclA determinant, including the promoter, ribosome binding site, and the first 35 codons of the open reading frame (ORF), was cloned into the E. coli-Bacillus shuttle plasmid pH13 (20). The resulting plasmid, pHHY4283, contains an in-frame XhoI site for fusing foreign genes to the BclA N-terminal targeting and attachment sequences. The bclA determinant is expressed off a ars promoter, so the fusion protein is not expressed in E. coli but only in B. thuringiensis during sporulation. Thus, expression of proteins that may be toxic to either E. coli or B. thuringiensis is feasible since there would be little expression during times when the bacteria are actively replicating. The bclA N-terminal domain (NTD) sequences will direct the fusion protein to the developing exosporium. The fusion protein will be cleaved after the 19th amino acid with attachment (presumably covalent) to the basal layer of the exosporium (16, 18, 21, 22). The fusion protein-decorated spores can then be easily purified away from residual proteins of the B. thuringiensis mother cell. The advantage of the BclA-based expression system over that of the B. subtilis-based systems, or that of the InhA1 (metalloprotease)-fusion system of B. thuringiensis (23), is the density of recombinant protein that can be truly displayed on the spore surface. To visualize the process of how the fusion protein is incorporated into developing spores, the mCherry protein ORF was cloned in-frame with the bclA targeting domain, expressed using the native bclA promoter, and found to be fully displayed on the spore surface (Fig. 2B). The BclA-mCherry fusion protein appears in the mother cell cytoplasm, is directed around the developing exosporium layer of the spore and concomitantly disappears from the mother cell cytoplasm, and then gets stably incorporated, in distinct steps. This provides a visual perspective of the amount of AtzA fusion protein that would be displayed on the spore surface. To evaluate this spore display system as a tool for bioremediation, we
cloned the atzA open reading frame into pHHY4283 to generate pHHY4290. This plasmid was introduced into B. thuringiensis, and spores were produced and evaluated for ATR chlorohydrolase activity.

**Activity of recombinant spores in water.** The enzymatic activity of AtzA-bearing B. thuringiensis spores was determined in the reaction with ATR (initial concentration of 5 mg liter\(^{-1}\)) in water. B. thuringiensis 4Q2-81 spores lacking the atzA determinant were used as a negative control. Four milligrams of spores was applied to the reactions, and more than 80% of the applied ATR in the reactions was enzymatically hydrolyzed into the much less toxic and less mobile metabolite HA by the AtzA-bearing spores within 24 h (Fig. 3). No detectable production of HA was observed with the 4Q2-81 control spores. The production of HA corresponding to the degradation of ATR in the reactions demonstrated that ATR was hydrolyzed to HA by the enzymatic activity of the BclA N-terminal domain-AtzA fusion protein displayed on the spore surface. The activity of the AtzA enzyme in catalyzing the dechlorination of ATR to HA (6) was not lost with the addition of the 22-amino-acid BclA targeting domain (residues 20 to 35 following the cleavage after amino acid 19 [18] and the 6-alanine linker). Furthermore, the physical anchoring of the fusion protein to the exosporium basal layer at its N terminus did not result in loss of enzymatic activity.

**Enzyme kinetics of the spore-tethered AtzA.** The enzyme kinetics of the spore-bound AtzA was determined by reactions of 1 mg of AtzA-bearing spores with various concentrations of ATR in a time course of 96 h at room temperature (Fig. 4). The results were plotted and calculated using the Hanes-Woolf equation. The enzymatic kinetics of AtzA-bearing spores was calculated to be a \(K_m\) of 132.2 \(\mu\)M and \(V_{\text{max}}\) of 0.96 nmol of HA h\(^{-1}\) mg spore\(^{-1}\) compared to the reported values of free recombinant AtzA protein, which exhibited a \(K_m\) of 149 \(\mu\)M and \(V_{\text{max}}\) of 2.6 \(\mu\)mol of HA min\(^{-1}\) mg protein\(^{-1}\) (6). Using a calibration curve of recombinant AtzA protein enzymatic activity (not shown), we determined that 1 mg of AtzA-bearing spores expressed activity equivalent to that of \(3.6 \times 10^{-4}\) mg of purified recombinant AtzA protein. After normalization, a \(V_{\text{max}}\) of 2.67 \(\mu\)mol of HA hour\(^{-1}\) mg protein\(^{-1}\) was obtained for the recombinant AtzA protein in this study.
Although the actual number of AtzA fusion proteins on the surface of the spores cannot be calculated, the activity of the spores can be compared to the activity equivalent of free enzyme. Given the molecular weight of AtzA (52,421 g/mol) and the weight of a B. thuringiensis spore (501.9 femtograms), each spore exhibits an activity equivalent to \( \frac{2.076 \times 10^3}{1.1003} \) AtzA enzyme molecules. This calculation is based on \( 1.99 \times 10^9 \) spores in 1 mg of a wet pellet, which is the spore titer per milligram that we measured. The number of AtzA molecules expressed on 1 mg of the spores is almost 4 magnitudes less than that of 1 mg of AtzA protein. That would explain the difference between the \( V_{max} \) values of the AtzA spore and AtzA protein. Spores decorated with AtzA demonstrated strong activities over the range of ATR concentrations tested (1 to 20 mg liter\(^{-1}\)).

**Time course studies with the AtzA-decorated spores in water.** ATR degradation assays in water were performed over a time course of 196 h at room temperature with the reactions consisting of 1 mg of AtzA-bearing spores or of the parent spores as controls (Fig. 5). The results of the time course study of degradation in water demonstrated that the ATR was degraded down to 58% by the 192nd hour with the AtzA-decorated spores. No discernible degradation was observed with the control spores (Fig. 5A). The decline in ATR to 66% of input occurred more rapidly during the first 72 h of incubation. A more gradual decrease was observed from the 72nd hour to the 192nd-hour end of the experiment. The activity had not plateaued at the 192nd-hour time point. The production of the metabolite HA was increased to approximately 35% (of the applied ATR) by the 192nd hour with AtzA-bearing spores, but no production of HA was observed with control spores (Fig. 5B). The appearance of HA correlated with the decline of ATR.

**Time course experiments in soil.** ATR degradation assays in soil were performed over a time course of 12 weeks with AtzA-bearing spores and recombinant AtzA proteins. B. thuringiensis 4Q2-81 spores were used as a control to monitor possible loss of ATR due to adsorption to spore surfaces. A background control, sterile soil without spore or protein addition, was included to monitor possible natural loss of ATR due to evaporation. The amounts of spores and free AtzA added to soil were adjusted to amounts that gave equivalent activity in the water experiments. Within 48 h, more than 80% of the applied ATR was degraded by AtzA-bearing spores, but only less than 20% of ATR was degraded by the recombinant AtzA protein. HA was the predominant degradation product in the reactions. All of the applied ATR was converted to HA by AtzA-bearing spores within 2 weeks of incubation. However, the degradation of ATR and the production of HA by AtzA protein only achieved 80% of ATR degradation at the end of the time course (Fig. 6).
Stability of the spore-expressed AtzA activity. To determine the stability of enzymes on the spore surface, an ATR degradation assay was performed using a batch of AtzA-bearing spores prepared in 2012 and stored in phosphate-buffered saline (PBS) at 4°C in comparison to another batch from 2016 that was prepared a week before the study. The spores prepared in 2014 resulted in 49% of the ATR being converted to HA, compared to a 58% reduction in ATR levels with the freshly prepared spores of 2016 (Fig. 7). Wild-type spores lacking AtzA were used as a control in this test, and they exhibited no detectable degradation of ATR. The enzymatic activity of the AtzA-bearing spores was only reduced by 18% over 45 months, suggesting an excellent shelf life of the spore platform with AtzA.

Spores versus free enzyme leaching simulation in soil experiments. To determine the leaching of enzyme proteins or spores in the soil, a leaching study was performed using soil packed in columns with a liquid application after the addition of recombinant AtzA enzyme or spores to mimic the natural leaching condition. ATR was applied to the soil
after the treatments and reacted with the remaining protein or spores, and the ATR degradation and the HA production as a function of the treatment with enzyme protein or spores were determined (Fig. 8). Following the washing treatment, the AtzA protein-supplemented soil lost 39% of the HA production compared to the unwashed soil. The HA production in the spore-inoculated soil after washing treatment was reduced by 30% relative to that without the washing treatment step.

Equal enzymatic activity of AtzA protein or AtzA-bearing spores (based on liquid assays) was applied in each treatment. However, the AtzA free protein lost 65% of its enzymatic activity when applied in the soil compared to that of the spore-bound enzyme (Fig. 8). This suggests that the spore-tethered enzymes may be more resistant to denaturation or other inhibitory conditions in the soil environment than free enzyme. This, in part, would explain the reduced overall activity of the free enzyme in the soil experiments of Fig. 6.

DISCUSSION

Bioremediation is the process that utilizes the metabolic capacity of microorganisms to reduce, eliminate, or contain contaminants present in soils, sediments, water, and air or transform them to benign compounds. Bioremediation of ATR in drinking water sources and contaminated soils, such as runoffs from ATR-applied fields or accidental spillage, is difficult and expensive in the absence of biological approaches. Even with
the field application of bacteria capable of degrading ATR, substantial detoxification of
the herbicide required multiple applications of the bacteria (25). Enzymatic bioremediation
is a potentially attractive method of removing environmental pollutant residues. However, enzymes or other catalytical agents for field bioremediation need to meet several critical criteria. First, they need to be stable and active under various physical and chemical environmental conditions. Second, they need to be resistant to proteolysis, changes in pH, changes in temperature, and the presence of inhibitors. Finally, a low overall cost associated with the production and purification of enzymes used in the remediation is a desirable property (8). There are approaches to improve properties of enzymes and enhance their catalytic rate, stability, and specificity of

FIG 6 Time course of atrazine degradation in soil with AtzA-bearing spores (red) and recombinant AtzA protein (blue). The percentage of atrazine or its metabolite, hydroxyatrazine, as a function of reaction time. ○, percentage of atrazine; ▲, percentage of hydroxyatrazine. The error bars show the standard deviations of the means of three independent duplicate determinants. Some of the error bars not shown are due to the standard deviation being < 0.01%.

FIG 7 Stability of the AtzA enzymatic activity expressed by AtzA-bearing *B. thuringiensis* spores. The AtzA-expressing spores were kept at 4°C in PBS before testing. One batch was generated and kept at 4°C since November 2012, and the other batch was generated in July 2016, a week before this assay was performed. The error bars show the standard deviations of the means of three independent duplicate determinants.
substrates, such as genetic engineering of recombinant proteins that stabilizes the active conformation of the protein or the technologies immobilizing enzymes onto supporting platforms (26, 27). However, these approaches inevitably require expensive and time-consuming recombinant protein production and purification or complicated immobilization processes that can have detrimental effects on enzymatic activity (7, 28).

Bacillus endospores are highly resistant to many physical and chemical assaults and are capable of long-term persistence in soil. Using B. thuringiensis endospores as a microparticle platform, we introduced degradative enzymes into contaminated soils, and the tethered enzymes were found to outperform the free enzymes. AtzA-decorated spores could detoxify ATR to nontoxic HA from both water and soil environments without the addition of exogenous salts or other cofactors.

The kinetic values found for the spore-bound AtzA, $K_m$ of 132.2 $\mu$M and $V_{max}$ of 0.96 nmol of HA hour$^{-1}$ mg spore$^{-1}$ and the reported values of free recombinant AtzA protein, which exhibited a $K_m$ of 149 $\mu$M and $V_{max}$ of 2.6 $\mu$mol of HA min$^{-1}$ mg protein$^{-1}$ (6), differed principally in the $V_{max}$. The calculated catalytic constant, $K_{cat}$, for the spores is substantially lower than that for enzymes in free form. In light of catalysts with low $K_m$ values performing better for soil remediation purposes due to their stronger affinity to contaminants (29), the spore particle would be more efficient catalytically in the field than the free enzymes. Enzyme stability is enhanced with the immobilized spore platform, especially in the soil. Most free enzymes have a half-life of less than 7 days after being applied in the field (C.-H. Lin [CSIRO], personal communication). Moreover, unlike other engineered free-enzyme systems, the spore system can be easily removed or recovered and reused, where applicable, in water bioremediation applications. Because the sporulating cells stably incorporate the bioremediation enzyme onto the spore surface, no expensive and time-consuming protein purification steps are required. This, coupled with the increased functional half-life of the incorporated enzymes, would make this approach a cost-effective approach to bioremediation of ATR and potentially other environmental pollutants.

**FIG 8** Degradation of atrazine in soils after leaching simulation with AtzA-bearing spores and recombinant AtzA protein. The percentage of atrazine or its metabolite, hydroxyatrazine, as a function of treatment. Solid color, treatments without leaching; stippled color, treatments with leaching. The error bars show the standard deviations of the means of three independent determinations of ATR performed in duplicate. Control refers to soil without enzyme or spore addition.
The substantially higher ATR degradation rates in soil than in water observed in this study were mainly attributed to metal cofactors present in the soils. AtzA is active as a hexamer that contains a trimer of dimers with essential Fe$^{2+}$ per monomer (30, 31). One of the key mechanistic features of the amidohydrolase superfamily is that many of these enzymes contain mononuclear or binuclear metal centers and the metal coordination geometry is essential for catalytic activity. Therefore, these metal ion cofactors, such as cobalt (II), iron (II), and zinc (II), often play crucial catalytic roles. Previous studies have also demonstrated that ATR hydrolysis by AtzA is strongly activated by iron(II) and cobalt(II) salts (30, 31). However, the water-based assays described herein were performed without metal salt supplementation. This suggests that the AtzA protein that is tethered to the spore surface has sufficient flexibility to multimerize with adjacent fusion proteins and function without additional cofactor supplementation. A requirement to multimerize for activity would also explain why the amount of active enzyme on the spores is lower than the amount of protein expected to be displayed on the spore surface (Fig. 2). The higher activity observed with the AtzA-bound spores following the washing steps in comparison with AtzA protein (Fig. 8) might result from a better distribution of the spores in the soil, which may result in more efficient interactions with the added ATR. Nonetheless, the experiment indicated there is 70% of spore-associated activity maintained under these leaching conditions.

This unique B. thuringiensis spore display system can potentially be used as a cost-effective enzyme delivery platform for quickly removing the persistent environmental pollutants. Although this initial study focused on AtzA and the BclA NTD, there are other anchoring spore collagen-like glycoprotein NTDs (18), so a single spore could express multiple degradative enzymes. However, it would be simpler to achieve a more complete degradation of ATR using a mixture of spores, each expressing a different Atz enzyme using the BclA NTD. In this mixture of spores, the proportion of each kind will be determined by the kinetics of its expressed enzyme, thus achieving an efficient and complete degradation of the herbicide. Future studies will be focused on the applications in a greenhouse setting and later in contaminated site studies. In addition, the system will be further evaluated against other classes of persistent environmental organic pollutants.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** *Pseudomonas* sp. strain ADP was grown in an atrazine-enriched medium (5, 32) at 30°C overnight with agitation. *E. coli* (DH5α or GM48) was cultured in Luria-Bertani (LB) broth for the gene cloning and protein production procedures. *B. thuringiensis* subsp. *israelensis* IQ2-81 (33), a plasmidless crystal toxin-negative strain, was obtained from the Bacillus Genetic Stock Center (Columbus, Ohio) and cultured in brain heart infusion (BHI) broth (Difco). Modified G broth (18) and nutrient agar (Difco) were used to examine sporulating cells and produce spores, respectively. When required, media were supplemented with 100 mg liter$^{-1}$ ampicillin or 10 mg liter$^{-1}$ chloramphenicol.

**Fluorescence microscopy.** Samples from sporulating cells in modified G broth were collected at indicated intervals. Images were obtained using a Nikon E600 epifluorescence microscope with an mCherry filter set using a ×60 or ×100 oil immersion objective.

**DNA extractions.** Overnight cultures of *Pseudomonas* sp. strain ADP or the plasmid-containing *E. coli* strains were harvested by centrifugation, and DNA extraction of plasmids was accomplished with the Promega Wizard Plus SV miniprep DNA purification system kit (Thermo Fisher). The Promega Wizard Genomic DNA purification kit (Thermo Fisher) was used to isolate total DNA from the *B. thuringiensis* strains.

**Cloning of the atzA and mCherry determinants.** Plasmid pADP-1 DNA was isolated from an overnight culture of *Pseudomonas* sp. strain ADP. The atzA determinant was PCR amplified using primers 5′-ctcgagATGCAAACGCGCTCAGCTCCGAGGAAGCGCTG-3′ and 5′-ctcgagCTAGAGGCTGAGCCAAGCTGGG-3′, which incorporated XhoI sites at their 5′ ends (shown in lowercase letters). The PCR cycling conditions were 94°C for 3 min and 35 cycles of 94°C for 30 s, 57.8°C for 30 s, 64.3°C for 30 s, and 72°C for 90 s. The 1.42-kb amplified fragment was cloned into the StrataClone PCR cloning vector pSC-A-amp/kan plasmid (Agilent Technologies) and the nucleotide sequence of the cloned fragment verified at the University of Missouri DNA Core Facility. The monomeric mCherry open reading frame (ORF) was similarly amplified with the primer pair 5′-ctcgagATGAAATCCGATGTCTTAGGG-3′ and 5′-ctcgagTATTATTATATATATATATTGGG-3′ to generate the BclA-NTD-mCherry reporter plasmid pS4260.

**Construction of the spore expression vector.** A spore expression vector was created in the pHP13 shuttle plasmid (20). A DNA fragment containing the bclA y$^{+}$ promoter, ribosome binding site, and the first 35 codons (including the BclA localization and attachment domains (16), followed by 6 alanine
codons, was cloned into pXmal as an Xmal to PstI fragment to create the plasmid pHHY4283. This plasmid has an in-frame Xhol site for fusing foreign genes to the BclA N-terminal targeting and attachment sequences. When the chimeric gene is expressed in sporulating cells of B. thuringiensis, the foreign protein will be displayed on the surface of the spore at high density. The atzA open reading frame was cloned in-frame into pHHY4283 as an Xhol fragment. The correct nature of the insert was verified by DNA sequence analysis, and the plasmid was designated pHHY4290.

**Electroporation of B. thuringiensis.** An overnight culture of B. thuringiensis was grown in BHI broth with 0.5% glycerol. We used 0.5 ml of this overnight culture to inoculate 100 ml of BHI plus 0.5% glycerol in a 500-ml Erlenmeyer flask. The culture was incubated at 37°C with shaking until the optical density at 600 nm (OD600) reached 0.6. The bacteria were then harvested by passage through a disposable analytical test filter funnel apparatus with a pore size of 0.45 μm (Nalgene). The cells were then thrice washed with 25 ml of ice-cold electroporation buffer (1 mM HEPES, 10% glycerol, pH 7.0). After the third wash, the filtered cells were recovered in 5 ml of ice-cold electroporation buffer and placed on ice. We loaded 1-mm electroporation cuvettes (Midwest Scientific) with 100 μl of cells and 1 μg of high-quality pHHY4290 plasmid DNA obtained from the GM48 dam-E. coli host. After loading, the cuvettes were pulsed at 25 μF, 200 ohm, and 2.25 kV. Immediately after pulsing, the cell/DNA mixture was resuspended in 1 ml of BGGM (BHI with 10% glycerol, 0.4% glucose, and 10 mM MgCl2). After incubation at 30°C for 2 h, aliquots were plated onto brain heart infusion agar (BHIA) plates containing chloramphenicol and incubated overnight at 30°C.

**B. thuringiensis spore preparation.** The AtzA-bearing spores were generated by culturing B. thuringiensis bearing pHHY4290 in LB broth with chloramphenicol at 10 mg liter⁻¹, incubated at room temperature with agitation for 48 h (resulting in > 95% spores), and harvested by centrifugation. The spore-containing pellets were repeatedly washed with PBS to remove bacterial cell debris. Spore amounts were normalized to 100 mg ml⁻¹ in sterile PBS and stored at 4°C.

**AtzA protein production and purification.** The complete nucleotide sequence of the approximately 1.9-kb DNA fragment containing the promoter region and AtzA ORF (6) was PCR amplified using pADP-1 as the template and cloned in pSC-A (StrataClone), creating pHHY4180. E. coli DH5α carrying pHHY4180 was grown at 37°C in 1 liter of LB broth containing 100 μg ml⁻¹ of ampicillin for 4 h after the OD600 reached 0.8. The culture was centrifuged at 3,500 × g for 10 min at 4°C. The cell pellet was resuspended in 25 mM morpholinepropanesulfonic acid (MOPS) buffer, pH 6.9, and cells lysed by bead beating with 0.1-mm glass beads for 2 min at 4°C. The cell crude extract was collected after centrifugation at 10,000 × g for 15 min, and solid NH₄SO₄ was added to the supernatant to reach 20% saturation. The precipitation was incubated for 8 h at 4°C with stirring and then centrifuged at 12,000 × g for 20 min. The precipitate was resuspended in 25 mM MOPS, pH 6.9, and dialyzed against 25 mM MOPS, pH 6.9, at 4°C. The protein preparation was retrieved after dialysis and used for experiments in this study.

**Enzymatic activity assay with spores and proteins.** The ATR stock solution was prepared with 10 mg liter⁻¹ (46 μM) of ATR (Millipore Sigma) in ultrapure water. AtzA-bearing spores or recombinant AtzA protein prepared in 20 μl of 25 mM MOPS, pH 6.9, was added into a brown glass bottle with 0.48 ml of ultrapure H₂O and 0.5 ml of ATR stock solution to achieve a final ATR concentration of 5 mg liter⁻¹ in the 1-ml reactions. The reaction mixtures were incubated at 25°C for 24 h with gentle agitation. We added 0.5 ml of methanol to extract the remaining ATR and its metabolite, HA. The extracted compound mixtures were filtered with the aid of a Whatman 0.2-μm Anotop syringe membrane filter (Thermo Fisher) and transferred to a high-performance liquid chromatography (HPLC) vial. The vials were kept at -20°C until chemical analysis with HPLC-triple quadrupole mass spectrometry (HPLC/MS/MS).

**Enzymatic activity assay with spores and proteins.** ATR concentrations of 4.6 μM, 23.2 μM, 46 μM, and 92.7 μM were used as the substrate for enzyme-bearing spores, and the reaction mixtures were incubated at 25°C and terminated at 0, 2, 6, 12, 24, 48, and 96 h by the addition of methanol. One milligram of spores in 20 μl of 25 mM MOPS, pH 6.9, was applied to each reaction. After incubation, methanol was added to extract the remaining ATR and its metabolite. HA. The extracted compound mixtures were filtered and analyzed by HPLC/MS/MS. The enzyme kinetics was determined following the procedure described by Shapir et al. (34). Catalytic kinetics were calculated using the Hanes-Woolf equation.

**Time course study in water.** The ATR stock solution was prepared with 10 mg liter⁻¹ (46 μM) of ATR in ultrapure water. One milligram of spores resuspended in 20 μl of 25 mM MOPS (pH 6.9) was added into a brown bottle with 0.48 ml of ultrapure H₂O and 0.5 ml of ATR stock solution to achieve a final ATR concentration of 5 mg liter⁻¹ in the 1-ml reactions. The samples, in duplicate, were incubated at 25°C for the time course with gentle agitation. Samples were collected at 0, 24, 48, 72, 120, and 192 h. At each time point, 0.5 ml of methanol was added into each sample and control to stop the reaction, and the spores were removed by centrifugation. One milliliter of each supernatant was filtered and analyzed by HPLC/MS/MS.

**Time course study in soil.** The ATR stock solution was prepared as 10 mg liter⁻¹ in ultrapure water. Sterile soil was composed of 60% sand and 40% Mexico silt loam (fine, smectitic, and mesic Vertic Epiaquolls) (33); it was autoclaved at 121°C for 1 h and oven-dried (55°C) for sterilization. One milligram of AtzA spores or wild-type spores resuspended in 1 ml of 25 mM MOPS, pH 6.9, or AtzA recombinant protein with activity equal to that of 1 mg of AtzA spores prepared in 1 ml of 25 mM MOPS, pH 6.9, was added into a brown bottle with silicon cap containing 1 g of sterile soil that had been pretreated with 1 ml of ATR stock solution for at least 2 h. Soil samples were set up for time points of 0, 1, 2, 4, 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77, and 84 days in duplicate. Each treatment and control were duplicated for each time point. The reaction mixtures were incubated at 25°C in the dark. At each time point, 5 ml of 100% methanol was added to each sample to stop the reaction, and the mixtures were transferred to

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15-mL centrifuge tubes. Sonication for 1 h was applied to all the samples to ensure complete extraction of ATR and its metabolite from soil. The first supernatant was collected after centrifugation at 5 × g for 5 min. Another 3 mL of 100% methanol was added to the pelleted soil, and sonication was applied again to the mixture for the extraction of remaining ATR and its metabolite in the soil. The second supernatant was collected after centrifugation and combined with the first one. Two milliliters of each pooled extract was filtered and analyzed by HPLC/MS/MS.

The simulation of the natural leaching process using columns. The experiments were performed based on the method of Shirav and Robl (36). The ATR stock solution and the soil were prepared as described above. Two milligrams of AtzA spores resuspended in 0.1 mL of 25 mM MOPS, pH 6.9, recombinant AtzA protein with activity equal to that of 2 mg of AtzA spores prepared in 0.1 mL of 25 mM MOPS, pH 6.9, or 0.1 mL of 25 mM MOPS, pH 6.9, as the control was added into a Bio-Rad Poly-Prep chromatography column containing 1 g of sterile soil in duplicate for leaching treatment or without the treatment. Five milliliters of 25 mM MOPS, pH 6.9, was applied to each column and drained by gravity immediately after leaching simulation or at the time when spore/protein was applied to the mixture. The reactions were stopped and processed as described above for the time course study in soil.

Chemical analyses. ATR and HA in the solutions and soils were extracted with 80% methanol. The extracts were filtered by Whatman 0.2-μm Anotop syringe membrane filters before the chemical analysis. The concentrations of ATR and the degradation products were determined using a Waters Alliance 2695 HPLC system coupled with a Waters Acquity TQ triple quadrupole mass spectrometer (HPLC/MS/MS) and a Waters 996 photodiode array detector (PDA). The phenolic compounds were chromatographically separated by a Phenomenex (Torrance, CA, USA) Kinetex C18 (100 mm by 4.6 mm; 2.6-μm particle size) reverse-phase column. The mobile phase consisted of 10 mM ammonium acetate and 0.1% formic acid in water (A) and 100% acetonitrile (B). The gradient conditions were 0 to 0.5 min, 2% B; 0.5 to 7 min, 2 to 80% B; 7 to 9 min, 80 to 98% B; 9 to 10 min, 2% B; and 10 to 15 min, 2% B at a flow rate of 0.5 mL/min. The MS/MS system was operated using electrospray ionization (ESI) in the positive ion mode with a capillary voltage of 1.5 kV. The ionization source was programmed at 150°C, and the desolvation temperature was programmed at 750°C. The concentrations of ATR and HA were quantified by measuring the protonated ions [M+H]+ m/z 216, m/z 198 with UV wavelengths of 220 nm and 240 nm, respectively.

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