An Antifungal Polycyclic Tetramate Macrolactam, Heat-Stable Antifungal Factor (HSAF), Is a Novel Oxidative Stress Modulator in *Lysobacter enzymogenes*

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ABSTRACT Polycyclic tetramate macrolactams (PoTeMs) are a fast-growing family of antibiotic natural products found in phylogenetically diverse microorganisms. Surprisingly, none of the PoTeMs have been investigated for potential physiological functions in their producers. Here, we used heat-stable antifungal factor (HSAF), an antifungal PoTeM from *Lysobacter enzymogenes*, as a model to show that PoTeMs form complexes with iron ions, with an association constant (K_a) of $2.71 \times 10^6 \text{ M}^{-1}$. The *in vivo* and *in vitro* data showed formation of 2:1 and 3:1 complexes between HSAF and iron ions, which were confirmed by molecular mechanical and quantum mechanical calculations. HSAF protected DNA from degradation in high concentrations of iron and H_2O_2 or under UV radiation. HSAF mutants of *L. enzymogenes* barely survived under oxidative stress and exhibited markedly increased production of reactive oxygen species (ROS). Exogenous addition of HSAF into the mutants significantly prevented ROS production and restored normal growth in the mutants under the oxidative stress. The results reveal that the function of HSAF is to protect the producer microorganism from oxidative damage rather than as an iron-acquisition siderophore. The characteristic structure of PoTeMs, a 2,4-pyrrolidinedione-embedded macrolactam, may represent a new iron-chelating scaffold of microbial metabolites. The study demonstrated a previously unrecognized strategy for microorganisms to modulate oxidative damage to the cells.

IMPORTANCE PoTeMs are a family of structurally distinct metabolites that have been found in a large number of bacteria. Although PoTeMs exhibit diverse therapeutic properties, the physiological function of PoTeMs in the producer microorganisms had not been investigated. HSAF from *Lysobacter enzymogenes* is an antifungal PoTeM that has been subjected to extensive studies for mechanisms of biosynthesis, regulation, and antifungal activity. Using HSAF as a model system, we here showed that the characteristic structure of PoTeMs, a 2,4-pyrrolidinedione-embedded macrolactam, may represent a new iron-chelating scaffold of microbial metabolites. In *L. enzymogenes*, HSAF functions as a small-molecule modulator for oxidative damage caused by iron, H_2O_2 , and UV light. Together, the study demonstrated a previously unrecognized strategy for microorganisms to modulate oxidative damage to the cells. HSAF represents the first member of the fast-growing PoTeM family of microbial metabolites whose potential biological function has been studied.

KEYWORDS natural products, polycyclic tetramate macrolactams, *Lysobacter enzymogenes*, oxidative damage, iron binding

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Polycyclic tetramate macrolactams (PoTeMs) are a family of natural products with diverse therapeutic properties, including antibacterial, antifungal, antiprotozoa, and anticancer (1–9). Their structures share a characteristic 2,4-pyrrolidinedione (tetramate)-containing macrolactam and have been found in phylogenetically diverse bacteria. For example, ikarugamycin, frontalamides, clifednamides, pactamides, capsimycins, and carbamides were isolated from various species of *Streptomyces* (1, 3–5, 8–10). Heat-stable antifungal factor (HSAF) and several alteramides were reported from several *Lysobacter* strains (2, 11–15). Maltophilin and xanthobaccin were isolated from *Stenotrophomonas* strains (16, 17). Discodermide and cylindramide were from marine sponges (18, 19). Umezawamides were from a combined culture of *Umezawaea* sp. and mycolic acid-containing bacterium *Tsukamurella pulmonis* (20).

The biosynthetic gene clusters (BGCs) for several PoTeMs have been reported (2, 3, 5, 10–14). Although the chemical structures are complex, BGCs exhibit relative simplicity and conserved organization. In the center of the BGC is always a single-module PKS-NRPS (polyketide synthase-nonribosomal peptide synthetase) hybrid gene, which is sufficient to construct the scaffold of PoTeMs (10–12, 14, 21–23). Flanking the PKS-NRPS gene are 2 to 6 accessory genes, which are responsible for the structural diversity of PoTeMs (14). Cryptic BGCs with this unique organization are present in numerous genome sequences in the databases, implying that there is an immense reservoir of PoTeM types of natural products yet to be discovered from the vast number of microorganisms (3, 9, 10). The therapeutic properties, structural novelty, diverse bioactivities, and distinct biosynthetic mechanism have attracted a lot of research interest in recent years. However, essentially nothing is known about PoTeMs' physiological functions in their producer organisms.

Many antibiotic metabolites are produced by microorganisms inhabiting diverse environments. In their native environments, the metabolites typically do not function as antibiotics to kill or inhibit other microorganisms because the producers rarely produce inhibitory concentrations of the metabolites in their environments (24). Many factors in the environment could affect the metabolite production and stress response in microorganisms. For example, reactive oxygen species (ROS) are stimulated in microorganisms growing in a high-iron environment or other stressed environments. Bacteria have evolved several strategies to modulate the oxidative stress induced by a high ROS level. The thioredoxin (Trx) system (NADPH, thioredoxin reductase, and thioredoxin) is a crucial antioxidant system in bacteria. The system removes ROS by providing electrons to thiol-dependent peroxidases. In most Gram-negative bacteria, the glutaredoxin system (Grx) and catalase provide a strong backup for the Trx system (25). Some catalase-negative bacteria such as *Streptococcus pyogenes* mainly utilize the thiol-dependent peroxidase system in defense against oxidative stress, although both Trx and Grx exist (26). Also, carotenoids and the aryl polyene-type bacterial pigments are proven to protect bacteria from ROS, which is related to their conjugation double-bond systems (27–29). Recently, the H₂S-mediated mechanism was found to protect against oxidative stress in *Escherichia coli* (30). The endogenous H₂S produced by 3-mercaptopyruvate sulfurtransferase sequesters free iron, which is necessary for the genotoxic Fenton reaction (30).

In this study, we have used the small-molecule metabolite HSAF from *Lysobacter enzymogenes* as a model PoTeM to explore its potential physiological functions. HSAF and alteramides (Fig. 1) isolated from *L. enzymogenes* are arguably the most extensively investigated PoTeMs in terms of structural diversity, bioactivities and modes of action, and molecular mechanisms for biosynthesis (2, 6, 7, 11–14, 31). Here, our results showed that the characteristic structure of PoTeMs, a 2,4-pyrrolidinedione-embedded macrolactam, can act as a new iron-chelating natural product scaffold. HSAF functions as a small-molecule modulator for oxidative damage caused by iron, H₂O₂, and UV light in *L. enzymogenes*. Together, the study demonstrated a previously unrecognized strategy for microorganisms to modulate oxidative damage to the cells.

FIG 1 Chemical structure of HSAF and analogs (alteramides) isolated from *Lysobacter enzymogenes*.

RESULTS

Formation of brown-orange complexes between HSAF and iron. During the study of *L. enzymogenes* OH11, we serendipitously found that adding iron salts into minimal culture media could make OH11 grow more robustly (Fig. S1a in the supplemental material). When we investigated the effect of different concentrations of FeSO_4 (0, 1, 10, 100, and 500 μM) on the growth of OH11 in a modified minimal medium (M813m) (Fig. S1b), we observed formation of brown-orange substances in both the cultures and the HSAF extracts (Fig. 2a and b). This color was intensified with the increase of the FeSO_4 concentration and was absent in the culture or extracts from an HSAF mutant (ΔHSAF) (32), even when grown in M813m containing 500 μM FeSO_4 . The result suggested that HSAF and iron ions might be able to interact with one another and form certain pigment complexes in the culture of OH11. Moreover, we found FeSO_4 could significantly boost the production of HSAF and alteramides (Fig. S2). With 1 to 10 μM FeSO_4 in M813m, the amount of free HSAF increased about 10- to 14-fold compared to that in the medium without FeSO_4 .

To verify the HSAF-iron complexes, we extracted the total PoTeM mixture containing both HSAF and its analogs (alteramides) from the cultures. When the mixture was added into an aqueous solution of FeSO_4 , the solution turned to the brown-orange color, with a gradually increased intensity following the increase of the PoTeM mixture, while the controls remained colorless (Fig. 2c). Furthermore, the same color could be developed in the PoTeM mixture when added with other iron salts, such as $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$, FeCl_3 , and $\text{Fe}(\text{NO}_3)_3$ (Fig. 2d). High-pressure liquid chromatography (HPLC) analysis of the mixtures showed that the PoTeM peaks significantly decreased or disappeared when any of the iron salts were added to the solutions (Fig. 2e). The interaction between PoTeM and iron ions appeared to be specific because HPLC showed that the PoTeM peaks remained in the solutions if the mixture was added

FIG 2 Formation of PoTeM-Fe complexes. (a) Appearance of cultures of *L. enzymogenes* OH11 wild type (WT) and HSAF nonproducer mutant (Δ HSAF) strains grown in M813m medium containing different concentrations of FeSO_4 . (b) Appearance of the total crude extract of PoTeM (200 μl) from cultures (25 ml) of WT and Δ HSAF grown in M813m containing different concentrations of FeSO_4 . (c) Color change in the solution of FeSO_4 (10 mM, 100 μl) when added with the total crude extract of PoTeM (1 to 50 μl , 2 $\mu\text{g}/\mu\text{l}$) from WT cultured in M813m containing 10 μM FeSO_4 , with methanol as a negative control. I, positive control (crude extract of PoTeM from WT grown in M813m containing 500 μM FeSO_4). (d) Color change in the solution of various iron salts (10 mM, 50 μl), without (0) or with 50 μl methanol (II) or with 50 μl (2 $\mu\text{g}/\mu\text{l}$) of the total crude extract of PoTeM (III). (e) HPLC analysis of the isolated PoTeMs, with or without iron salts added.

with other metal ions (Na^+ , Mg^{2+} , K^+ , Ca^{2+} , and Zn^{2+}), although there might have been some interactions between PoTeMs and Cu^{2+} (Fig. S3).

Mass spectrometry of HSAF-Fe complexes. The above-described observations indicated that HSAF and its analogs could form complexes with iron ions. To obtain direct evidence, we purified HSAF from the OH11 culture and treated HSAF (10 mM) with the same concentration of aqueous FeSO_4 , $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$, FeCl_3 , or $\text{Fe}(\text{NO}_3)_3$. The products were analyzed by mass spectrometry (MS). Without the iron ion, all MS gave two main peaks, m/z 513 for $[\text{HSAF}+\text{H}]^+$ and m/z 1,025 for $[2\text{HSAF}+\text{H}]^+$, which are expected for standard HSAF (Fig. 3). Upon treatment with the iron ion, the HSAF peaks markedly decreased [when added with ferrous ion, FeSO_4 , or $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$] or disappeared [when added with ferric ion, FeCl_3 , or $\text{Fe}(\text{NO}_3)_3$]. In the meantime, two new peaks appeared (m/z 1,079 and 1,591) when treated with FeSO_4 or $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ (Fig. 3a and b), or just one new peak appeared (m/z 1,079) when treated with FeCl_3 or $\text{Fe}(\text{NO}_3)_3$ (Fig. 3c and d). The peak at m/z 1,079 is coincident with $[2\text{HSAF}-\text{H}+\text{Fe}]^+$, whereas the peak at m/z 1,591 is coincident with $[3\text{HSAF}-\text{H}+\text{Fe}]^+$. The data showed that two or three HSAF molecules could coordinate with one iron ion to form stable HSAF-Fe complexes that displayed the observed orange-brown color. The data also suggested that all HSAF chelated Fe when a ferric salt was used, as seen with FeCl_3 and $\text{Fe}(\text{NO}_3)_3$, but only a portion of HSAF chelated iron when a ferrous salt was used, as

FIG 3 MS analysis of the products of HSAF with FeSO_4 (a), $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ (b), FeCl_3 (c), and $\text{Fe}(\text{NO}_3)_3$ (d) in the absence or presence of the metal chelator EDTA. Standard HSAF gave an m/z 513 for $[\text{HSAF}+\text{H}]^+$ and m/z 1,025 for $[2\text{HSAF}+\text{H}]^+$. In the mixtures of HSAF and iron salts, the peak at m/z 1,079 was coincident with $[2\text{HSAF}-\text{H}+\text{Fe}]^+$, whereas the peak at m/z 1,591 was coincident with $[3\text{HSAF}-\text{H}+\text{Fe}]^+$.

seen with FeSO_4 and $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ (Fig. 3). Since ferrous iron can gradually be oxidized to a ferric ion in the atmosphere, it is likely that the observed partial chelation of FeSO_4 and $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ was due to the oxidized iron (ferric). In addition, both $2\text{HSAF}-\text{Fe}$ and $3\text{HSAF}-\text{Fe}$ were observed when ferrous salts were used, whereas only $2\text{HSAF}-\text{Fe}$ was detected when ferric salts were used. This also is evidence that ferric ion is the preferred iron for HSAF chelation because the concentration of ferric ion in solution would be lower when FeSO_4 and $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ were used than that when FeCl_3 and $\text{Fe}(\text{NO}_3)_3$ were used, and thus, the HSAF concentration was relatively high, and two or three HSAF molecules were available to chelate one ferric ion in FeSO_4 and $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ solutions. To further confirm the formation of HSAF-Fe complexes, EDTA, a strong chelator for metal ions, was added into the mixtures of HSAF and iron salts. MS clearly showed that the HSAF-Fe complexes (m/z 1,079 and 1,591) were abolished and HSAF (m/z 513 and 1,025) was restored (Fig. 3).

Absorbance spectra of HSAF-Fe complexes. Next, we analyzed the absorbance spectra of the HSAF-Fe complexes (Fig. S4). Standard HSAF gave a maximum peak at ~ 323 nm, which shifted to ~ 310 nm upon addition of any of the iron salts, FeSO_4 , $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$, FeCl_3 , or $\text{Fe}(\text{NO}_3)_3$. While HSAF or the iron salts barely had any absorption in the visible range, the HSAF-Fe complexes gave clear absorptions at ~ 410 to 600 nm, which apparently contributed to the orange-brown appearance of the mixtures. Furthermore, the maximal absorption shifted back to 323 nm from 310 nm, and the absorption at ~ 410 to 600 nm disappeared upon addition of EDTA (Fig. S4). The absorption spectroscopic data are in accordance with that of the MS analysis. Using

FIG 4 The *in vitro* antioxidant activity and the *in vivo* protective effect of HSAF for *L. enzymogenes* grown in the presence of H_2O_2 . (a) *In vitro* deoxy-D-ribose degradation assay for the antioxidant activity of HSAF. The activity is presented as thiobarbituric acid reactive species (TBARS) levels relative to the control (100% = TBARS of the control reaction mixture without HSAF). (b) *In vitro* assay of the Fenton reaction-caused DNA fragment degradation and the protective effect of HSAF. (c to e) *In vivo* H_2O_2 sensitivity assay of the WT and ΔHSAF strains cultured in M813m medium containing 0 (c), 80 (d), or 800 (e) μM H_2O_2 , and the OD₆₀₀ value was determined every 24 h. Data are presented as averages of three independent experiments, each conducted in triplicate. *, $P < 0.05$; **, $P < 0.01$.

the UV-visible titration of HSAF with $\text{Fe}(\text{NO}_3)_3$ and nonlinear curve fitting at 470 nm, we found the association constant (K_a) of HSAF-Fe to be $2.71 \times 10^6 \text{ M}^{-1}$ (Fig. S5). The K_a value is much smaller than that for recognized siderophores (33), indicating that, rather than function as a siderophore for iron acquisition from the environment, HSAF in *L. enzymogenes* may play a new role during the interaction with iron ion.

Antioxidant activity of HSAF and protection of DNA degradation *in vitro*. To understand possible roles of HSAF in *L. enzymogenes*, we explored the potential involvement of HSAF in the modulation of oxidative stress because it is well-known that the cellular iron could generate reactive oxygen species (ROS) due to the Fenton reaction, which can lead to cell death (34). The *in vivo* and *in vitro* data described above showed the formation of HSAF-Fe complexes, which might contribute to maintaining a proper free iron concentration, which is important to redox homeostasis of the bacterial cells.

To test this hypothesis, we used the deoxyribose degradation assay to determine HSAF's antioxidant activity (35). HSAF showed dose-dependent antioxidant activity and behaved as a strong antioxidant when the concentration reached 80 to 160 μM (Fig. 4a). The reaction system for the assay contained deoxy-D-ribose, H_2O_2 , Fe^{3+} , ascorbic acid, and purified HSAF. To exclude possible direct interactions between HSAF and H_2O_2 , ascorbic acid, or deoxy-D-ribose, we analyzed the mixtures using HPLC. As expected, HSAF was not affected by any of the factors (H_2O_2 , ascorbic acid, or deoxy-D-ribose) (Fig. S6). The results indicated that the antioxidant activity of HSAF resulted from the chelation with iron ion. To confirm the antioxidant function of HSAF, we carried out *in vitro* DNA fragment degradation caused by Fenton reaction due to production of the radical species (36). A DNA fragment with a length of 1 kb was amplified by PCR using the genome of *L. enzymogenes* as the template. The DNA fragment was

completely degraded in the presence of Fe^{3+} and H_2O_2 , while the DNA fragment remained intact in the controls. However, the addition of purified HSAF inhibited the DNA degradation in a dose-dependent manner (Fig. 4b). When HSAF reached $80\ \mu\text{M}$, the DNA fragment was fully protected from the Fenton reaction-caused degradation, which is in good agreement with the antioxidative activity assay (Fig. 4a). To learn whether this protective effect of HSAF is specific to certain DNA fragments, we tested similar-length DNA fragments from *Lysobacter* strain 3655, *Lysobacter antibioticus* OH13, and *Escherichia coli*. HSAF exhibited similar protective effects against the Fenton reaction-caused damage on these DNA fragments, showing a general antioxidative effect of HSAF (Fig. S7).

Protection of *L. enzymogenes* OH11 from high H_2O_2 stress by HSAF *in vivo*. The observation that HSAF can protect *in vitro* DNA degradation by H_2O_2 inspired us to investigate HSAF's function *in vivo*. The wild-type (WT) and ΔHSAF strains were treated with different concentrations of H_2O_2 in M813m medium. The results clearly showed that the growth rates of the WT and ΔHSAF were similar when culture medium contained a low concentration of H_2O_2 ($80\ \mu\text{M}$), but in the medium containing a high concentration of H_2O_2 ($800\ \mu\text{M}$), the growth rate of the ΔHSAF strain significantly decreased compared to that of the WT (Fig. 4c to e). Actually, the ΔHSAF strain did not grow in M813m containing $800\ \mu\text{M}$ H_2O_2 in the first 48 h and started to grow slowly only at 72 h, while the WT grown in M813m containing $800\ \mu\text{M}$ H_2O_2 could reach optical density at 600 nm (OD_{600}) values similar to those of the WT without H_2O_2 at 72 to 96 h. We also analyzed the HSAF level in cultures containing 0, 80, and $800\ \mu\text{M}$ H_2O_2 . This showed that the production of HSAF/alteramides in the WT with $800\ \mu\text{M}$ H_2O_2 decreased by 30% compared to that of the WT with 0 or $80\ \mu\text{M}$ H_2O_2 , implying consumption of HSAF for formation of HSAF-Fe complexes to protect the cells grown in a high concentration of H_2O_2 (Fig. S8). To exclude the possibility that HSAF in the WT could directly degrade H_2O_2 so that the WT could grow in a high concentration of H_2O_2 , we tested the ability of HSAF and HSAF-Fe complexes to degrade H_2O_2 *in vitro* (Fig. S9). The results show that H_2O_2 was not degraded by either HSAF or HSAF-Fe complexes. Furthermore, MS analysis of the ethyl acetate extract from the WT culture clearly detected the peaks at m/z 1,079 for $[\text{2HSAF-H+Fe}]^+$ and 1,591 for $[\text{3HSAF-H+Fe}]^+$, in addition to m/z 513 for $[\text{HSAF+H}]^+$ (Fig. S10). This showed HSAF-Fe complexes were formed *in vivo*.

Protection of *L. enzymogenes* OH11 from high iron stress *in vivo*. Next, we looked into the effect of HSAF on the growth yield of *L. enzymogenes* OH11 under different concentrations of iron ion. The WT and ΔHSAF strains in regular M813m medium containing $10\ \mu\text{M}$ iron ion exhibited growth yields similar to those in M813m medium without iron ion (Fig. S11a and b). However, in M813m containing a high concentration ($500\ \mu\text{M}$) of iron ion, the growth yield of the ΔHSAF strain was significantly lower than that of the WT after 72 to 120 h of growth, although the two strains exhibited similar growth yields in the first 48 h (Fig. S11c). The addition of HSAF to the cultures after 48 h of growth restored the growth yield of the ΔHSAF strain to the WT level in the following 72 to 120 h of growth, even when the iron concentration was as high as $500\ \mu\text{M}$ (Fig. S11d). The results show that HSAF can protect OH11 cells from high iron stress *in vivo*. In addition, the exogenously added HSAF-Fe complexes, but not HSAF alone, could promote the growth of the WT strain in M813m medium without supplemented FeSO_4 (Fig. S12a). However, neither HSAF nor HSAF-Fe complexes affect the growth of the WT strain in regular M813m medium containing FeSO_4 (Fig. S12b). The results suggested that the iron ion could be released from HSAF-Fe complexes and then taken up by the cells to support the observed growth promotion.

Protection of *L. enzymogenes* OH11 from UV radiation by HSAF. In light of HSAF's protection of *L. enzymogenes* from oxidative damage induced by high concentrations of iron and H_2O_2 , we looked into the protective role of HSAF when the cells were exposed to UV radiation because UV exposure can also lead to a variety of ROS through various mechanisms (37). When the WT and ΔHSAF strains were exposed to UV light for a short time (10 s), the survival rates of the WT and ΔHSAF cells were

FIG 5 Protective effect of HSAF for *L. enzymogenes* exposed to UV light and ROS level in *L. enzymogenes* under oxidative stress. (a) WT and ΔHSAF strains were exposed to UV light for 0, 10, 30, and 60 s, and the numbers of colonies on each plate were counted after 72 h of incubation at 30°C. (b) Rescue of the UV light sensitivity of the ΔHSAF strain by exogenous HSAF. The ΔHSAF strain was added with various amounts of HSAF (0, 20, 80, and 160 μM) and then exposed to UV light for 60 s. (c) ROS level in the WT and ΔHSAF strains cultured in different media. No Fe, M813 minimal medium without FeSO₄; M813m, M813 modified medium containing 10 μM FeSO₄; High Fe, M813 modified medium containing 500 μM FeSO₄. (d) ROS level in the WT and ΔHSAF strains treated with 40 mM H₂O₂. (e) ROS level in the WT and ΔHSAF strains treated with UV light for 60 s. Methanol was used as a control. Data are presented as averages of three independent experiments, each conducted in triplicate. *, *P* < 0.05; **, *P* < 0.01.

similar. However, the survival rate of ΔHSAF cells was significantly decreased when the UV exposure time increased to 30 s, and there were nearly zero colonies on the plate with 60 s of UV exposure, while the WT still had a large number of colonies (Fig. 5a; Fig. S13a). On the other hand, the exogenous addition of purified HSAF to the ΔHSAF culture before the UV exposure could restore growth. The rescue of the ΔHSAF cells by HSAF was clearly dose dependent, and when the HSAF concentration reached 160 μM, the survival rate of the ΔHSAF strain was even higher than that of the WT (Fig. 5b; Fig. S13b). The results unequivocally showed the protective effect of HSAF on cells exposed to UV radiation.

Repression of ROS accumulation in *L. enzymogenes* OH11 by HSAF. Next, we tested ROS formation in the strains by the method using H₂DCFDA (2',7'-dichlorodihydrofluorescein diacetate), which is a cell-permeant indicator that generates fluorescence after oxidation by ROS (38). The results showed almost no detectable ROS in the WT and ΔHSAF strains when cultured in the minimal medium without iron ion and, similarly, a low level of ROS production in the WT and ΔHSAF strains in the modified medium containing a low level of iron (M813m containing 10 μM FeSO₄) (Fig. 5c). In a high-iron medium (M813m containing 500 μM FeSO₄), the ΔHSAF strain produced nearly twice as much ROS as the WT, although the ROS level increased significantly in both the WT and ΔHSAF strains. Remarkably, when exogenous HSAF was added to the ΔHSAF strain grown in the 500-μM FeSO₄ medium, the ROS level returned to the WT level. The results clearly showed that HSAF is able to repress the high-iron-caused ROS production (Fig. 5c). Moreover, the ROS level in ΔHSAF was about 2-fold higher than

that in the WT when treated with H_2O_2 or UV light, and the exogenous HSAF restored the WT level of ROS in the ΔHSAF strain (Fig. 5d and e). Next, we directly measured the ROS scavenging activity of HSAF and HSAF-Fe complexes using DPPH (2,2-diphenyl-1-picrylhydrazyl), which is a stable radical and has a maximum absorption at 520 nm. The results showed that HSAF and HSAF-Fe complexes exhibited low ROS scavenging activity when the incubation time was 0.5 h, while with the extension of incubation time (24 h, 72 h, and 120 h), the ROS scavenging activity of both HSAF and HSAF-Fe complexes increased (Fig. S14a and b). As expected, ascorbic acid showed strong ROS scavenging activity (Fig. S14c). This is consistent with the observation in the UV irradiation assay where the culture time was 72 h and HSAF exhibited ROS scavenging activity. The data clearly showed the ROS-modulating ability of HSAF in *L. enzymogenes*.

Molecular structure of HSAF-Fe complexes. To obtain further evidence for formation of the HSAF-Fe complexes, we performed molecular mechanical and quantum mechanical calculations to determine the possible molecular structures of the HSAF-Fe complexes. Using a molecular mechanical force field method, a global search suggested that both Fe^{3+} and Fe^{2+} ions can be chelated by 2 or 3 neutral HSAF molecules. The quantum mechanical method was used to refine the molecular geometries (Fig. 6). When 2 molecules of HSAF bind to an iron ion, the 3 carbonyl oxygen atoms (at C-7, C-25, C-27; see Fig. 1) of each of the two HSAF molecules form 3 coordinate bonds with the iron. When 3 molecules of HSAF bind to an iron ion, two HSAF molecules provide the oxygen atoms at C-7 and C-27, and the third HSAF molecule provides the oxygen atoms at C-25 and C-27. Together, the three HSAF molecules form 6 coordinate bonds with the iron. Due to steric factors, it would be impossible for 4 molecules of HSAF to bind to one iron. The carbonyl oxygen atoms at C-7, C-25, and C-27 are absolutely conserved in all PoTems, suggesting that formation of such iron complexes is general for all PoTems. The chelation status is similar to that of the *Pseudomonas* quinolone signal (PQS) with an iron ion, in which two or three PQS molecules chelated one iron ion (39).

DISCUSSION

Since the isolation of HSAF and its analogs from *Lysobacter enzymogenes*, research has focused on their antifungal activity, as well as the molecular mechanisms for their biosynthesis and regulation. The work presented here attempts to address the role of these complex molecules in their producer organism. *Lysobacter* species are emerging as a rich source of bioactive natural products. During our efforts to activate silent biosynthetic gene clusters in the genomes of *Lysobacter* species, we serendipitously found that the addition of iron salts enhanced HSAF production. Meanwhile, we observed the formation of a brown-orange color when iron salts were added to the cultures that produced HSAF and its analogs, but not in the biosynthetic mutant. *In vitro* studies using the crude extracts and purified HSAF confirmed that the color was due to formation of complexes between these compounds and iron. The results also showed that the production of HSAF and its analogs is essential for *L. enzymogenes* to survive under oxidative conditions that are known to generate reactive oxygen species (ROS).

When *L. enzymogenes* was exposed to a high concentration of iron, H_2O_2 , or UV radiation, the HSAF biosynthetic mutant was barely able to survive, while the wild type and the mutant supplemented with HSAF exogenously could grow normally. In bacteria, ROS are induced by many stresses, including the presence of a high iron concentration, H_2O_2 treatment, and UV radiation (40). Indeed, we observed a significantly higher level of ROS production in the HSAF mutant than in the wild type. The supplement of HSAF to the mutant reduced the ROS level to that of the wild type.

We also observed that HSAF protects DNA from degradation in the presence of iron and H_2O_2 , probably due to hydroxyl radicals ($\cdot\text{OH}$) generated by Fenton reaction (equation 1). The hydroxyl radical is a highly reactive ROS and is able to oxidize practically every molecule in the cell (41, 42).

FIG 6 Molecular structure of HSAF-Fe complexes obtained from the molecular mechanical force field method and quantum mechanical method. Two molecules (a) or three molecules (b) of HSAF can chelate one iron ion.

In both prokaryotes and eukaryotes, the oxidative DNA damage caused by hydroxyl radicals is the primary cause of cell death under oxidative stress conditions (43–45). We thus hypothesized that HSAF may be involved in the Fenton reaction and affect the

redox homeostasis of *L. enzymogenes*. In bacteria, both $O_2^{\cdot-}$ and H_2O_2 are primarily produced by the accidental autoxidation of nonrespiratory flavoproteins, which are univalent electron donors giving electrons to oxygen (46). In addition, a high iron concentration also could promote H_2O_2 generation. In the deoxyribose degradation assay, ascorbic acid initializes the Fenton reaction by reducing Fe^{3+} to Fe^{2+} , and Fe^{2+} , in turn, reacts with H_2O_2 to generate ROS ($\cdot OH$) (equation 1) (35). In the *in vitro* assay of DNA fragment degradation, Fe^{3+} could be reduced to Fe^{2+} through reacting with H_2O_2 by following the two-step reaction (equations 2 and 3) (47). The reactions generate Fe^{2+} and then ROS by the reaction between Fe^{2+} and H_2O_2 , as shown in equation 1.

As shown in Fig. 2, HSAF prefers ferric ion over ferrous ion in the chelation. The HSAF- Fe^{3+} chelation would prevent the reduction of Fe^{3+} to Fe^{2+} in the two-step reaction (equations 2 and 3). Consequently, the sequestration of Fe^{3+} ion by HSAF would result in a reduced amount of free Fe^{2+} in the cells and, in turn, reduced ROS from the Fenton reaction. The K_a value of HSAF-Fe complexes is significantly smaller than that of typical siderophores. This suggests that a relatively high concentration (micromolar level) of HSAF would be needed in order to form stable complexes with iron. HSAF and alteramides are the predominant secondary metabolites in *L. enzymogenes* (2). We were able to obtain up to 50 mg HSAF from 1 liter of culture, suggesting that the concentration of HSAF in the cells would be higher than $100 \mu M$. Thus, the concentration at which HSAF exhibited iron chelation and strong antioxidant activity *in vitro* could be readily achievable *in vivo*.

Moreover, we showed that the exogenous addition of HSAF into the culture media could restore the Δ HSAF mutant's growth in a high concentration of iron or H_2O_2 or with exposure to UV radiation; we also showed that the exogenous HSAF was able to make the ROS level in the Δ HSAF mutant return to the WT level, even when grown in the $500 \mu M$ $FeSO_4$ medium. Together, these observations implied that extracellular HSAF was able to enter the cells. However, the exact mechanism of HSAF transportation is not totally clear at the moment. Beyond chelating iron, HSAF can also scavenge ROS directly, as seen in Fig. 5c to e and Fig. S14a and b in the supplemental material. These results explain the protective effect of HSAF on *Lysobacter* with UV exposure (Fig. 5a and b).

Although HSAF is able to chelate iron, the HSAF-Fe complexes form only when the iron concentration is sufficiently high (above the micromolar level). This is in contrast to siderophores, whose primary function is to grab metal ions from environments where the concentration of the metals can be extremely low. HSAF functions as a modulator for oxidative stress only when the cells are exposed to an environment with a high concentration of iron or H_2O_2 or are exposed to UV light, all of which can lead to generation of ROS, which damage DNA and affect cell survival.

Moreover, our study using methods in molecular mechanical force field and quantum mechanics indicated that the carbonyl groups at C-7, C-25, and C-27 of the HSAF structure are involved in the formation of the HSAF-Fe complexes. These carbonyl groups are absolutely conserved in all PoTeMs. This finding is significant because it suggests that formation of such iron complexes could occur for all PoTeMs. On the other hand, there is a clear structural diversity among the PoTeM family, which is derived from the polycyclic system (14). While HSAF and alteramides are known for antifungal activity, other members of the PoTeM family exhibit antitumor, antiprotazoal, cytotoxic, and antiviral activities. The structural diversity of PoTeMs may be associated with these activities and may also confer a survival advantage to their producers in various habitats. For example, *L. enzymogenes* OH11 was originally isolated from the rhizosphere of a pepper plant (32). Whether HSAF and alteramides play a role in plant root colonization is worth further investigation, as resistance to oxidative stress is

TABLE 1 Bacterial strains used in this study

<i>Lysobacter</i> strains	Relevant characteristics ^a	Source or reference no.
<i>L. enzymogenes</i> OH11	Wild type, Kan ^r	CGMCC 1978
ΔHSAF	HSAF-deficient strain	32

^aKan^r, kanamycin resistant.

important for the survival of bacteria during their interaction with plants. It is not very clear what structural features in these compounds are associated with the antifungal activity, although we found the carbonyl oxygen atoms at C-7, C-25, and C-27 are involved in the iron chelation to form HSAF-Fe complexes. *L. enzymogenes* can produce HSAF and analogs without iron and with iron in minimal media (up to 500 μ M). In reality, it is unlikely that the iron concentration in the natural environment of *L. enzymogenes* would be higher than what we have tested. Several iron chelators have exhibited antifungal activity, such as siderophores produced by *Azospirillum brasilense* that could inhibit the growth of *Colletotrichum acutatum* and the siderophore oxachelin from *Streptomyces* sp. GW9/1258 that showed strong antimicrobial activity against several fungi and Gram-positive bacteria (48, 49). Further studies are needed in order to answer whether the antifungal activity and the antioxidant activity are related to each other or exclude each other. Nevertheless, it seems reasonable to assume that the antioxidant activity of HSAF and its analogs could enhance the survival rate of the producer microorganism through the enhanced resistance to oxidative stress and thus play a role in plant root colonization during its interaction with plants. Several strategies have evolved in bacteria to modulate oxidative stress (25–30).

In summary, HSAF has been recognized as an antifungal antibiotic with a fascinating chemical structure, new mode of action, and distinct mechanism for biosynthesis (2, 6, 7, 31). Here, we presented evidence to support that HSAF and its analogous compounds may represent a new strategy for microorganisms to modulate oxidative stress. These “secondary metabolites” allow *L. enzymogenes* to survive in an environment with a high concentration of iron, H₂O₂, or UV radiation. HSAF is the first member of the fast-growing PoTeM family of natural products whose potential biological function has been investigated. The genome-mining efforts have shown a strikingly conserved organization for PoTeM biosynthetic clusters, which are present in a large number of unexplored genomes of phylogenetically diverse bacteria ranging from proteobacteria to actinomycetes (3, 10, 14). This means there are a large number of new PoTeMs yet to be explored. Understanding the biological function of HSAF sheds new light on the critical role of secondary metabolites in the survival of microorganisms in complex ecosystems, and the results will also facilitate future efforts in new PoTeM exploitation.

MATERIALS AND METHODS

Bacterial cultures, primers, and PCR. The wild-type strain *Lysobacter enzymogenes* OH11 (CGMCC 1978) and the HSAF biosynthetic mutant ΔHSAF (Table 1) were cultured in Luria-Bertani (LB) medium (32). For the production of HSAF and its analogs, the strains were cultured in M813 modified medium (4 g glucose, 3 g K₂HPO₄, 1.2 g NaH₂PO₄, 1 g NH₄Cl, 0.3 g MgSO₄, 0.15 g KCl, 10 mg CaCl₂, and 2.8 mg FeSO₄·7H₂O per liter) (50). In the initial experiments, the strains were also cultured in MM2 medium (4 g glucose, 15 g KH₂PO₄, 34 g Na₂HPO₄, 5.4 g NH₄Cl, 2.5 g NaCl, 0.3 g MgSO₄, and 10 mg CaCl₂ per liter). Table 2 listed the primers used in this study. Phusion high-fidelity DNA polymerase (Thermo Scientific) was used as the amplification enzyme. The PCR started from an initial denaturation at 98°C for 30 s, followed by 30 cycles of amplification (98°C for 10 s, 60°C for 15 s, and 72°C for 1 min), and was completed with an additional 5 min at 72°C. Depending on the DNA templates and primers, the annealing temperature and the elongation time were adjusted in some cases.

Extraction and HPLC analysis of HSAF and its analogs. *L. enzymogenes* OH11 wild type (WT) and an HSAF-deficient strain (ΔHSAF), in which a part of the *pks-nrps* gene (from 232 bp to 1,356 bp relative to the start codon) of the HSAF biosynthetic gene cluster was deleted (32), were incubated in 1 ml LB at 30°C with shaking at 200 rpm overnight. An aliquot (1%) of the cultures was transferred to 25 ml M813 modified (M813m) medium with variable concentrations of FeSO₄ (final concentrations of 0, 1, 10, 100, and 500 μ M) at 30°C with shaking at 200 rpm for 48 h. The whole cultures (cells and medium) were treated with 75 μ l trifluoroacetic acid (TFA) and 25 ml ethyl acetate. The organic phase was dried with the airflow, and the residues were redissolved in 200 μ l methanol. A 2- μ l aliquot of each extract was analyzed by HPLC (Agilent; 1220 Infinity LC). Water plus 0.05% formic acid (FA) (solvent A) and acetonitrile

TABLE 2 Primers used in this study

Primer	Sequence (5'–3')
OH11-F	CGGGGCCCCATTGGAACGACAGCCTCTT
OH11-R	CCGCTCGAGCGGCAAGACAGGGGAAGA
3655-F	CGGGGCCCCCTTTGGTTGTTCCATCCGA
3655-R	CGGGATCCATCGAGGAGCACGGCATC
OH13-F	CTGCAGGACTTCGAACACA
OH13-R	GATTGACTCCTTGGTGCTC
E-F	ATAACGGAGAACGGAATCG
E-R	ACGCATTACTATCTGACCAA

plus 0.05% FA (solvent B) were used as the mobile phases with a flow rate of 1.0 ml/min. The HPLC program was as follows: 5 to 25% solvent B at 0 to 5 min, 25% to 80% solvent B at 5 to 25 min, 80 to 100% solvent B at 25 to 26 min, maintained to 28 min, back to 5% solvent B at 29 min, and maintained to 30 min. HSAF and its analogs were detected at 318 nm on a UV detector. For purification of HSAF, the WT was incubated in 10 ml LB at 30°C with shaking at 200 rpm overnight. An aliquot (1%) of the cell cultures was transferred to 1 liter M813m medium and grown at 30°C with shaking at 200 rpm for 48 h. The culture broth was adjusted to pH 2.5 with 37% HCl. To the culture was added the same volume of ethyl acetate, and HSAF was extracted into the organic phase three times. The ethyl acetate phase was dried using a rotavapor, and HSAF was separated from other metabolites in the extract (850 mg) on a C₁₈ reverse-phase column and eluted with different concentrations of methanol (10%, 30%, 50%, 70%, and 100%). The fraction (552 mg) of 100% methanol was used to purify HSAF (~50 mg) by HPLC.

Chelation of HSAF with metal ions. For the chelation with FeSO₄, an increased volume (1, 10, and 50 μ l in methanol) of the total PoTeM mixture (2 mg/ml) was mixed with 100 μ l aqueous solution of FeSO₄ (10 mM). For the chelation with other iron salts, the total PoTeM mixture (50 μ l, 2 mg/ml) was incubated with 50 μ l aqueous solution of Fe(NH₄)₂(SO₄)₂, FeCl₃, and Fe(NO₃)₃ (each 10 mM). For the chelation with other metals, the total PoTeM mixture (50 μ l; 0.5 mg/ml) was incubated with 50 μ l aqueous solution of Na₂SO₄, MgSO₄, K₂SO₄, Ca(NO₃)₂, CuSO₄, and ZnSO₄ (each 10 mM). Methanol was used as a control. For HPLC analysis, the above-mixed solutions were dried and then resuspended in 100 μ l methanol. A 20- μ l aliquot of each of the solutions was analyzed by HPLC (Agilent, 1220 Infinity LC). The HPLC program was as follows: water plus 0.05% FA (solvent A) and acetonitrile plus 0.05% FA (solvent B) were used as the mobile phases with a flow rate of 1.0 ml/min. The program was as follows: 5% to 60% solvent B at 0 to 5 min, 60% to 100% solvent B at 5 to 20 min, maintained to 23 min, back to 5% solvent B at 28 min, and maintained to 30 min. The metabolites were detected at 230 nm on a UV detector. The experiments were repeated three times.

MS analysis and UV-visible absorbance spectra of the HSAF-Fe complexes. To prepare the complexes, purified HSAF (20 μ l, 10 mM) was mixed with an equal volume of each of aqueous FeSO₄ (10 mM), Fe(NH₄)₂(SO₄)₂ (10 mM), FeCl₃ (10 mM), and Fe(NO₃)₃ (10 mM). Each of the mixtures was dried and resuspended in 200 μ l double-distilled water (ddH₂O). Ethyl acetate (200 μ l, containing 0.05% TFA) was added to the suspension to extract the HSAF-Fe complexes, and the organic phase was collected, dried, and redissolved in 1 ml methanol. A 20- μ l aliquot of each of the samples was analyzed by MS, and the remaining fraction of the samples was used to determine the UV-visible absorbance spectrum using a spectrophotometer (Shimadzu UV-Vis 2501). After the spectra were taken, 50 μ l Na₂EDTA (100 mM, pH 8.0) was added into each of the samples, and the samples were dried and resuspended in 250 μ l ddH₂O. Similar to the above procedure, the HSAF-Fe complexes were extracted with 250 μ l ethyl acetate (containing 0.05% TFA), and the organic phase was collected, dried, and redissolved in 1 ml methanol. Then, samples were similarly analyzed by MS and the spectrophotometer again. The experiments were repeated three times.

UV-visible absorbance titration and association constant determination. To determine the association constant, absorbance spectra were recorded with a spectrophotometer (Shimadzu UV-Vis 2501). The UV-visible titration assay was performed by using a constant host concentration of HSAF (0.1 mM) and variable concentrations of Fe(NO₃)₃ at 25°C. The association constant (K_a) was calculated using equation 4 by applying a nonlinear curve-fitting method (51) in the program Origin 9.0 to changes in absorbance (Δ Abs) at 470 nm.

$$\Delta \text{Abs} = \left\{ L(1 + K_a X + K_a A) - [L^2(K_a X + K_a A + 1)^2 - 4K_a^2 AXL^2]^{0.5} \right\} / 2K_a A \quad (4)$$

where X and A were the total concentrations of the guest and the host, respectively, and L and K_a were treated as parameters. The experiments were repeated three times.

Assay for deoxyribose degradation. This assay was used to determine the antioxidative activity. In the assay, hydroxyl radicals generated by the Fenton reaction would degrade deoxy-D-ribose into malonyldialdehyde (MDA) (35). MDA then would react with 2-thiobarbituric acid to produce a pink pigment, which was measured photometrically at 532 nm (34). To perform the assay, 227.5 μ l of purified HSAF (final concentration of 0, 5, 10, 20, 40, 80, or 160 μ M) was added into the reactant mixture containing 45.5 μ l deoxy-D-ribose (52 mM in 50 mM KH₂PO₄, pH 7.4), 91 μ l FeCl₃ (4 μ M in water), 91 μ l KH₂PO₄ buffer

(pH 7.4), 45.5 μl H_2O_2 (10 mM in water), and 45.5 μl ascorbic acid (1 mM in 50 mM KH_2PO_4 , pH 7.4). The samples were mixed and incubated at 30°C for 60 min. After that, 455 μl 2-thiobarbituric acid (1%, in 3% trichloroacetic acid) was added into each of the samples, and the mixtures were incubated at 85°C for 30 min. The supernatant of each of the mixtures was collected following centrifugation (12,000 rpm, 1 min), and the OD_{532} value of the supernatants was determined by spectrophotometer (Shimadzu UV-Vis 2501). The experiments were repeated three times.

Assay for *in vitro* DNA degradation. The assay followed a previously described method with some modifications (36). The DNA fragments were obtained by PCR using primers listed in Table 2 with a template from the genomic DNA of *L. enzymogenes* OH11, *Lysobacter* strain 3655, *L. antibioticus* OH13, or *E. coli*. Each of the degradation mixtures contained 100 ng DNA fragment, 20 mM H_2O_2 , 150 μM FeCl_3 , and various concentrations of purified HSAF (0.3125, 0.625, 1.25, 2.5, 5, 10, 20, 40, and 80 μM) in 50 mM KH_2PO_4 buffer (pH 7.4). After incubating the mixtures at 37°C for 60 min, the samples were applied to 0.8% agarose gels in Tris-acetate-EDTA (TAE) buffer. The electrophoresis was performed at 140 V for 15 min, and the bands were visualized in a UV transilluminator (Universal Hood; Bio-Rad). The experiments were repeated three times.

OH11 growth under oxidative stress induced by H_2O_2 . WT and ΔHSAF strains were incubated in 1 ml LB at 30°C with shaking at 200 rpm overnight. A fraction (1%) of the cultures was transferred to 25 ml M813m medium at 30°C with shaking at 200 rpm for 48 h. After the OD_{600} value of each of the cultures was determined, a fraction (1%) of the cultures was added into 25 ml M813m medium containing 0, 80, or 800 μM H_2O_2 , and the cultures were incubated at 30°C with shaking at 200 rpm for 96 h. The OD_{600} values of the WT and ΔHSAF strains were recorded every 24 h. The experiments were repeated three times.

Assay for *in vitro* H_2O_2 degradation. The hydrogen peroxide (H_2O_2) colorimetric assay kit (Elabscience, China) was used to determine the concentration of H_2O_2 which could react with ammonium molybdate to produce a yellow complex with a maximum absorption at 405 nm. The reaction system contained 2 ml reaction buffer, 100 μl H_2O_2 (60 mM), and 100 μl purified HSAF (4 mM) or 100 μl purified HSAF-Fe complexes (4 mM). Methanol was used as a negative control. The reaction system was incubated at 30°C for 30 min, and then the OD_{405} value was determined by spectrophotometer.

OH11 growth under oxidative stress induced by UV light. The WT and ΔHSAF *L. enzymogenes* OH11 strains were incubated in 1 ml LB at 30°C with shaking at 200 rpm overnight. A fraction (1%) of the cultures was transferred to 25 ml M813m medium at 30°C with shaking at 200 rpm for 48 h. The cultures were adjusted with the medium to the same OD_{600} of 1.5, and a fraction of 10 ml of each of the cultures was spread on a petri dish (9.0-cm external diameter) and exposed to a UV light source (253.7 nm; model TUV 30W T8; 102 V, 0.37 A, 30 W) at a distance of 30 cm between the light and the cells for 0 s, 10 s, 30 s, or 60 s. For the complementary assay, purified HSAF at various concentrations (final concentrations of 0, 20, 80, and 160 μM) was added into the culture of the ΔHSAF strain before exposure to UV for 60 s, and methanol was used as a control. The cultures were serially diluted and spread on fresh LB plates. The numbers of colonies on each plate were counted after 72 h of incubation at 30°C. The experiments were repeated three times.

ROS detection. The production of ROS in the WT and ΔHSAF strains was detected using a previous method with some modifications (38). The WT and ΔHSAF strains were incubated in 1 ml LB at 30°C with shaking at 200 rpm overnight. A fraction (1%) of the cultures was transferred into 25 ml M813m medium with various concentrations of FeSO_4 (0, 10, and 500 μM). After 72 h of growth, the cultures were diluted 30-fold with the same medium in a 96-well plate. Then, H_2DCFDA was added to the wells with a final concentration of 10 μM . The incubation of the plate continued in the dark at 30°C with shaking at 60 rpm for 6 h. Fluorescence was measured in a BioTek Synergy H1 plate reader (excitation, 495 nm; emission, 527 nm). In addition, the WT and ΔHSAF cultures from regular M813m medium containing 10 μM FeSO_4 were treated with UV for 60 s without or with purified HSAF (final concentration, 160 μM) or treated with H_2O_2 (final concentration, 40 mM) before fluorescence detection. The experiments were repeated three times.

Assay for ROS scavenging activity of HSAF and HSAF-Fe complexes. HSAF and HSAF-Fe complexes were tested for *in vitro* ROS scavenging activity using DPPH (2,2-diphenyl-1-picrylhydrazyl), which is a stable radical and has maximum absorption at 520 nm. In the reaction system, 1 ml DPPH (5 mg/ml, dissolved in ethanol) was mixed with 200 μl various concentrations of purified HSAF or HSAF-Fe complexes (final concentrations were 0 μM , 20 μM , 40 μM , 80 μM , and 160 μM , dissolved in ethanol) and then incubated at room temperature for 0.5 h, 24 h, 72 h, and 120 h. The OD_{520} values of samples were determined by a spectrophotometer, which was used to measure the remaining DPPH by following the formula $\text{DPPH (\%)} = A/A_0 \times 100\%$; A_0 represents the OD_{520} value of 0 μM . Ascorbic acid was used as a positive control, and the OD_{520} value was determined immediately.

Extraction of the HSAF-Fe complexes *in vivo*. The WT was incubated in 1 ml LB at 30°C with shaking at 200 rpm overnight. A fraction (1%) of the cultures was transferred into 50 ml M813m medium containing 500 μM FeSO_4 and incubated at 30°C with shaking at 200 rpm for 48 h. After centrifugation (12,000 rpm, 5 min), the precipitate presented as two layers, and the upper layer was collected and extracted with 50 ml ethyl acetate (containing 0.3% TFA). The organic phase was dried and redissolved in 200 μl methanol. The methanol solutions were used for MS analysis. The experiments were repeated three times.

Molecular mechanical and quantum mechanical calculations. The calculations were performed with the quantum chemistry polarizable force field (QuanPol) (52) program and the General Atomic and Molecular Electronic Structure System (GAMESS) package (53, 54). The Merck Molecular Force Field 94 (MMFF94) force field (55–58) was used in the global search of the most stable molecular structures. In

the global search, one million steps (time step size, 1 fs) of molecular dynamic simulation were performed at 700 K, with a geometry optimization every 1,000 steps. Using the MMFF94-identified minimum structures, quantum mechanical density functional theory method B3LYP (59, 60) (with Grimme's empirical dispersion correction version III [61]) was used to refine the molecular geometries. The 6-31G* basis set (62) was used.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.1 MB.

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The calculations were performed with resources at the University of Nebraska-Lincoln Holland Computing Center.

We have no conflict of interest to declare.

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