

Heme Proteins

Inhibition of Heme Uptake in *Pseudomonas aeruginosa* by its Hemophore (HasA_p) Bound to Synthetic Metal Complexes**

Chikako Shirataki, Osami Shoji,* Mitsuyoshi Terada, Shin-ichi Ozaki, Hiroshi Sugimoto, Yoshitsugu Shiro, and Yoshihito Watanabe*

Abstract: The heme acquisition system A protein secreted by *Pseudomonas aeruginosa* (HasA_p) can capture several synthetic metal complexes other than heme. The crystal structures of HasA_p harboring synthetic metal complexes revealed only small perturbation of the overall HasA_p structure. An inhibitory effect upon heme acquisition by HasA_p bearing synthetic metal complexes was examined by monitoring the growth of *Pseudomonas aeruginosa* PAO1. HasA_p bound to iron-phthalocyanine inhibits heme acquisition in the presence of heme-bound HasA_p as an iron source.

The heme acquisition system A (HasA) protein is a hemophore secreted by some pathogenic bacteria with a heme acquisition (Has) system such as *Pseudomonas aeruginosa*,^[1] *Serratia marcescens*,^[2] *Pseudomonas fluorescens*,^[3] and *Yersinia pestis*.^[3b,4] (Figure 1). Under the low-iron conditions found in animal cells, these bacteria secrete HasA to capture heme from their hosts as an iron source.^[2b] The heme captured by HasA is transferred to a hemophore-specific outer-membrane receptor called HasR.^[5] HasA-type hemophores

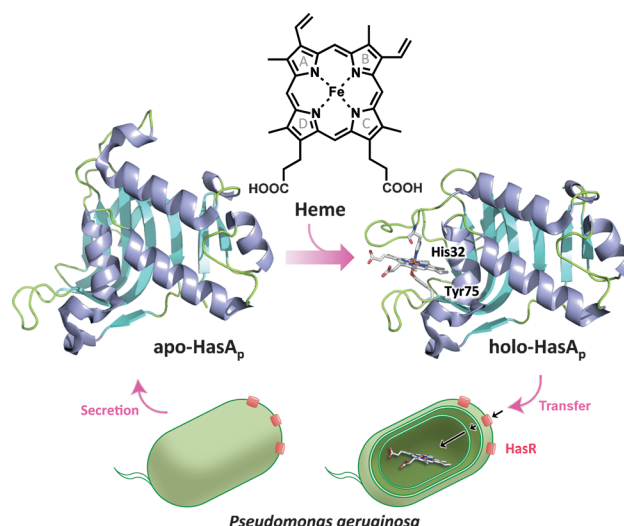


Figure 1. The Has system in *P. aeruginosa*. Under low-iron conditions, *P. aeruginosa* secretes apo-HasA_p (PDB ID: 3MOK), which captures heme (PDB ID: 3ELL); the resulting holo-HasA_p delivers the captured heme to the specific outer membrane receptor, HasR, on *P. aeruginosa*. In holo-HasA_p, His32 and Tyr75 coordinate to the heme iron atom. In the bound heme, the edges of the pyrrole rings A, C and D are highly solvent exposed.

[*] Prof. Dr. Y. Watanabe
Research Center for Materials Science, Nagoya University
Furo-cho, Chikusa-ku, Nagoya 464-8602 (Japan)
E-mail: p47297a@nucc.cc.nagoya-u.ac.jp
Homepage: <http://bioinorg.chem.nagoya-u.ac.jp/>
C. Shirataki, Dr. O. Shoji, M. Terada
Department of Chemistry, Graduate School of Science
Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8602 (Japan)
E-mail: shoji.osami@a.mbox.nagoya-u.ac.jp
Prof. Dr. S. Ozaki
Department of Biological Science, Yamaguchi University
1677-1 Yoshida, Yamaguchi 753-8515 (Japan)
Dr. H. Sugimoto, Prof. Dr. Y. Shiro
RIKEN SPring-8 Center, Harima Institute
1-1-1 Kouto, Sayo, Hyogo 679-5148 (Japan)

[**] This work was supported by Grants-in-Aid for Scientific Research (S) to Y. W. (24225004), Grant-in-Aid for Scientific Research on Innovative Areas "Molecular Activation Directed toward Straight-forward Synthesis" to Y. S. (22105012) and to O. S. (25105724), and a Grant-in-Aid for Young Scientists (A) to O. S. (21685018) from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) (Japan). C. S. was supported by Global COE Program "Elucidation and Design of Materials and Molecular Functions" and the Program for Leading Graduate Schools "Integrative Graduate Education and Research Program in Green Natural Sciences" by MEXT (Japan). We thank Dr. Go Ueno, Dr. Yuki Nakamura, Dr. Hironori Murakami, and Dr. Nobuyuki Shimizu for their assistance with the data collection at SPring-8.

Supporting information for this article is available on the WWW under <http://dx.doi.org/10.1002/anie.201307889>.

have attracted much attention for their heme acquisition from target hemoproteins such as hemoglobin in the hosts^[2b] and for the transfer of heme from HasA to HasR.^[6] The crystal structure of the heme-bound form (holo form) of the hemophore from *S. marcescens* (HasA_{sm}) was first solved by Arnoux et al.^[7] HasA_{sm} binds heme through two loops with coordination of Tyr75 and His32 to the heme iron center.^[7] The overall structure of HasA_{sm} bound to heme resembles "a fish biting the heme".^[7] Subsequently, Alontaga et al. reported the crystal structure of the hemophore from *P. aeruginosa* (HasA_p).^[8] The structures of these two hemophores are essentially identical.

The crystal structures of the holo forms of HasA proteins show that the captured heme moiety is highly solvent exposed; not only the propionate groups but also the edges of the heme pyrrole rings A, C, and D can be seen from outside (Figure 1). This unique fashion of heme binding, like a pair of tweezers gripping the heme, suggests that metal complexes with different structures to heme could be accommodated by HasA proteins. Because the heme-free form (apo form) of HasA exhibits an open conformation with a large rearrangement of the His-32 loop (Figure 1),^[9] even relatively bulky metal complexes could enter the heme

binding pocket. These considerations led us to investigate whether HasA could capture metal complexes other than heme. We were also interested in how the addition of synthetic metal complexes to HasA might affect the transfer of heme from HasA to HasR. We report herein that the hemophore from *P. aeruginosa* (HasA_p) can capture several metal complexes including Fe-phthalocyanine (Fe-Pc) without any structural perturbation and the resulting Fe-Pc-bound HasA_p markedly inhibits the growth of *P. aeruginosa* even in the presence of heme-bound HasA_p.

First, we investigated whether HasA_p interacts with synthetic metal complexes that have different structures to heme by subjecting a simple mixture of 3.5 μM apo-HasA_p and a methanolic solution of metal complexes (2.7 equiv) to electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS; (Figure 2a–e). With heme, the intensity ratio of apo-HasA_p to holo-HasA_p was 1:0.6 under the conditions examined (Cone voltage: 45 V). Under similar conditions, the synthetic metal complexes iron mesoporphyrin IX (Fe-MPIX), iron 2,3,7,8,12,13,17,18-octaethyl-21H,23H-porphyrin (Fe-OEP), and iron *N,N*-disalicylal-1,2-phenylenediamine (Fe-salophen) gave peaks corresponding to holo-HasA_p formation, whereas iron tetraphenylporphyrin (Fe-TPP) showed only the peak for apo-HasA_p (Figure 2a–e). The intensity of the peaks for holo-HasA_p bound to these metal complexes was comparable to or even larger than that measured with heme, thus indicating that the complexes of HasA_p with Fe-MPIX, Fe-OEP, or Fe-salophen are relatively stable^[10] (Figure 2a–e, the UV/Vis spectra of samples used for ESI-TOF-MS are shown in Figure S1 in the Supporting Information). In fact, we have succeeded in purifying HasA_p bound to these metal complexes by column chromatography.

UV/Vis spectra of HasA_p bound to metal complexes after purification are shown in Figure S2. A titration experiment showed 1:1 binding of HasA_p and the metal complexes (see Figure S3). These results suggest that HasA_p can capture these metal complexes, which have a planar structure, in the

same fashion as with heme. We have further succeeded in crystallizing HasA_p bound to Fe-salophen and Fe-MPIX (Figure 3a,b). The crystal structures show clear electron densities assignable to Fe-salophen (Figure 3d) and Fe-MPIX (Figure 3e) at the heme-binding position. The overall structures of Fe-salophen-bound HasA_p and Fe-MPIX-bound HasA_p are essentially identical to that of heme-bound HasA_p (root-mean-square deviation (RMSD) over 2–184 amino acid residues for Ca atoms: 0.810 for Fe-salophen and 0.443 for Fe-MPIX), whereas the His32 loop in Fe-salophen-bound HasA_p is clearly shifted inwards as a result of induced-fit binding to accommodate a small metal complex (Figure S4). Both Tyr75 and His32 bind to the iron atoms of Fe-salophen and Fe-MPIX. The location of Fe-MPIX is identical to that of heme but Fe-MPIX is captured in a reverse orientation relative to that of heme (minor orientation; Figure 3k and Figure S4), possibly as a result of steric repulsion between the two ethyl groups of Fe-MPIX, which protrude out of the porphyrin plane. This observation indicates that heme binding by HasA_p is regulated by recognition of the vinyl groups of the heme so that heme can only be bound in a single orientation. Although the structure of Fe-salophen is largely different from heme, Fe-salophen was similarly captured by HasA_p. The central phenyl ring of the salophen ligand was accommodated in the hydrophobic pocket composed of Phe46, Tyr56, Leu85, His134, Val137, and Met141 located deep in the heme binding site (Figure 3g). This interaction seems to be a key interaction in the binding of Fe-salophen and leads to a single orientation of Fe-salophen in HasA_p.

Given the crystal structure of HasA_p bound to Fe-salophen, it is intriguing to examine binding of Fe-phthalocyanine (Fe-Pc) to HasA_p because the structure of Fe-Pc is similar to that of Fe-salophen and the crystal structures shown in Figure 3 suggest that there is enough space to accommodate Fe-Pc, which bears a large π-plane. Phthalocyanine is a highly hydrophobic molecule with a large planar structure and is thus insoluble in water; it is even difficult to

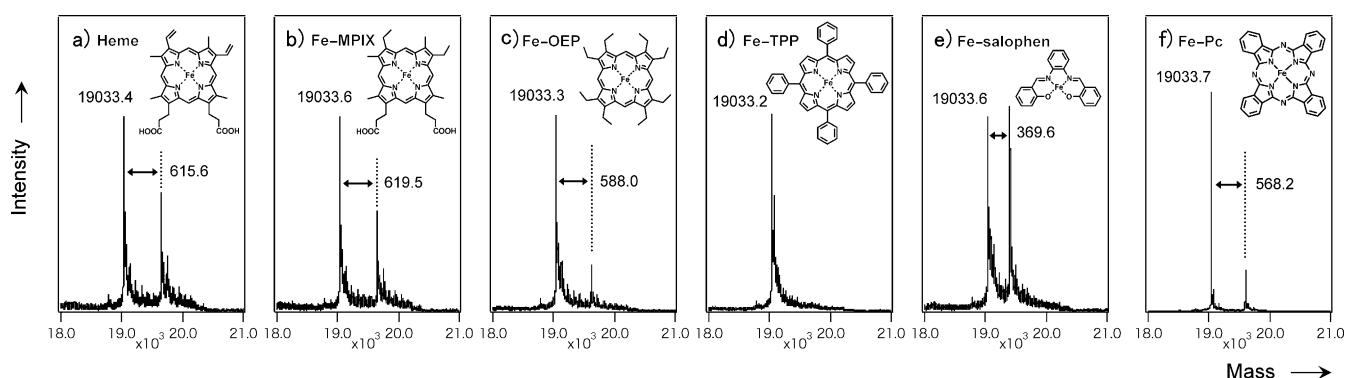


Figure 2. Positive mode ESI-TOF-MS spectra of the mixtures of apo-HasA_p and the metal complexes: a) hemin; b) Fe-MPIX-Cl; c) Fe-OEP-Cl; d) Fe-TPP-Cl; and e) Fe-salophen-Cl. The sample mixture was prepared by adding 100 μL (2.7 equiv) of metal complexes dissolved in methanol to a solution of purified apo-HasA_p in ammonium acetate buffer (5 mM, 3.5 nmol, 900 μL). Cone voltage was fixed at 45 V. The molecular weight of apo-HasA_p was calculated to be 19034.77 Da based on its amino acid sequence. f) Positive mode ESI-TOF mass spectrum of purified HasA_p harboring Fe-Pc in 5 mM ammonium acetate buffer. The concentration of HasA_p with Fe-Pc was 4.5 μM. The exact masses of the metal complexes are 616.18 Da (heme), 620.21 Da (Fe-MPIX), 588.29 Da (Fe-OEP), 668.17 Da (Fe-TPP), 370.04 Da (Fe-salophen), and 568.08 Da (Fe-Pc).

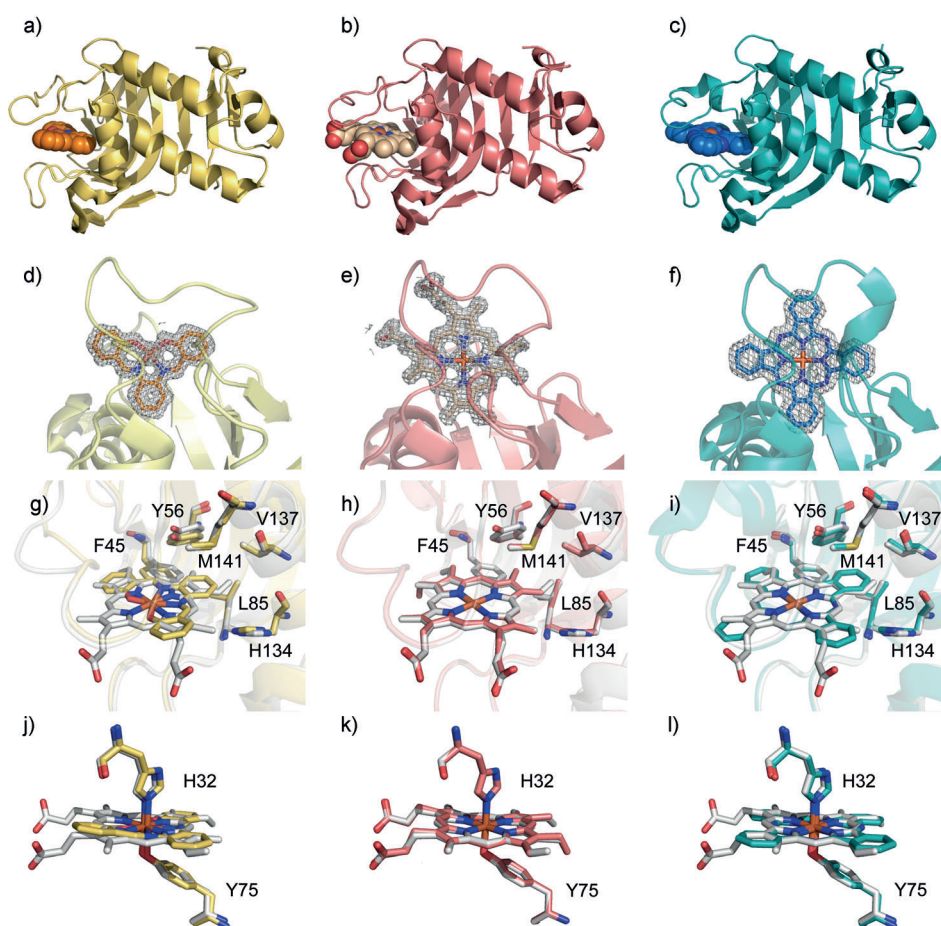


Figure 3. X-ray crystal structures of HasA_p with synthetic metal complexes: Fe-salophen (yellow; a, d, g, j); Fe-MPIX (pink; b, e, h, k); and Fe-Pc (turquoise; c, f, i, l). In the side views of overall structures (top row, a–c) HasA_p is shown as a ribbon model and the metal complexes as space filling models. In the top views of the binding sites (second row, d–f), HasA_p is shown as a ribbon model and $2F_o - F_c$ electron-density maps of the metal complexes contoured at the 1.0 σ level are shown in gray mesh. In the third row (g–i), close-up views corresponding to those from the propionate side of the heme are shown, with the metal complexes and the hydrophobic amino acid residues located in the binding pocket depicted as stick models. Close-up views of the side of the metal complexes are shown in the bottom row (j–l), with H32, Y75 and the metal complexes shown as stick models. In the bottom two rows, the crystal structure of heme-bound HasA_p (PDB ID: 3ELL) is superimposed on the stick models and depicted in light gray. The electron densities from $F_o - F_c$ Fourier synthesis omitting the metal complexes are presented in the Figure S5. The atomic coordinates and structure factors (codes 3W8M, 3WAH, and 3W8O) have been deposited in the Protein Data Bank.

dissolve Fe-Pc in methanol, mainly because of a strong stacking interaction. We thus had to use other polar organic solvents to dissolve the Fe-Pc. Because apo-HasA_p did not aggregate even in 50 % DMSO, we used a DMSO solution of Fe-Pc to examine the binding of Fe-Pc to apo-HasA_p. Fe-Pc-bound HasA_p was stable even after the removal of DMSO by dialysis (Figure 2 f, Figure S2). The crystal structure of Fe-Pc-bound HasA_p showed clear electron density for Fe-Pc and the binding fashion was similar to that of Fe-salophen (Figure 3 f). As was observed for Fe-salophen binding, one of the phenyl rings of Fe-Pc was accommodated in the hydrophobic pocket in the depths of the heme binding site (Figure 3 i). Only minor perturbation of the overall structure of HasA_p was observed (Figure 3 c and Figure S4). The RMSD over 2–

184 amino acid residues for C α atoms against heme-bound HasA_p was estimated to be 0.915.

To examine the effect of synthetic metal complexes on the growth of *P. aeruginosa*, we cultured *P. aeruginosa* PAO1 in the presence of HasA_p bound to the artificial metal complexes and monitored bacterial growth through measuring the absorbance of the culture at 600 nm (OD₆₀₀; Figure 4 a). Even though *P. aeruginosa* did not grow in a medium containing EDTA as an iron ion scavenger (Figure 4 a, green line), *P. aeruginosa* started to grow following the addition of heme-bound HasA_p to the medium (Figure 4 a, red line), thus indicating that *P. aeruginosa* uses the heme in holo-HasA_p as an iron source. Growth of *P. aeruginosa* was also observed following the addition of Fe-MPIX-bound HasA_p (Figure 4 a, pink line), thus indicating that *Pseudomonas aeruginosa* could utilize Fe-MPIX as an iron source. These results show that the transfer of heme from HasA_p to HasR as well as the heme degradation processes to acquire iron^[11] are not much affected by the substitution of the two vinyl groups for ethyl groups. By contrast, for HasA_p bound to Fe-salophen or Fe-Pc, the OD₆₀₀ values did not increase. This suggests either that Fe-salophen and Fe-Pc are not transferred to HasR or that they are transferred to HasR but the subsequent steps were inhibited (Figure 4 c, Steps III, IV, and V).

To examine the influence of Fe-salophen and Fe-Pc on the growth of *P. aeruginosa*, we cultured the bacteria in media containing both HasA_p bound to heme and HasA_p bound to Fe-salophen or Fe-Pc. Interestingly, in the presence of Fe-Pc-bound HasA_p, the OD₆₀₀ values did not increase at all despite the presence of heme-bound HasA_p (Figure 4 b, blue line). HasA_p bound to Fe-salophen did not affect the growth of *P. aeruginosa* under these conditions. These results clearly indicate that HasA_p bound to Fe-Pc inhibits heme acquisition by the Has system. The half-maximal inhibitory concentration (IC₅₀) was estimated to be 24 nM, thus indicating that Fe-Pc-bound HasA_p efficiently inhibited heme transportation (Figure S6). No toxicity for the cells of HasA_p bound to Fe-Pc was

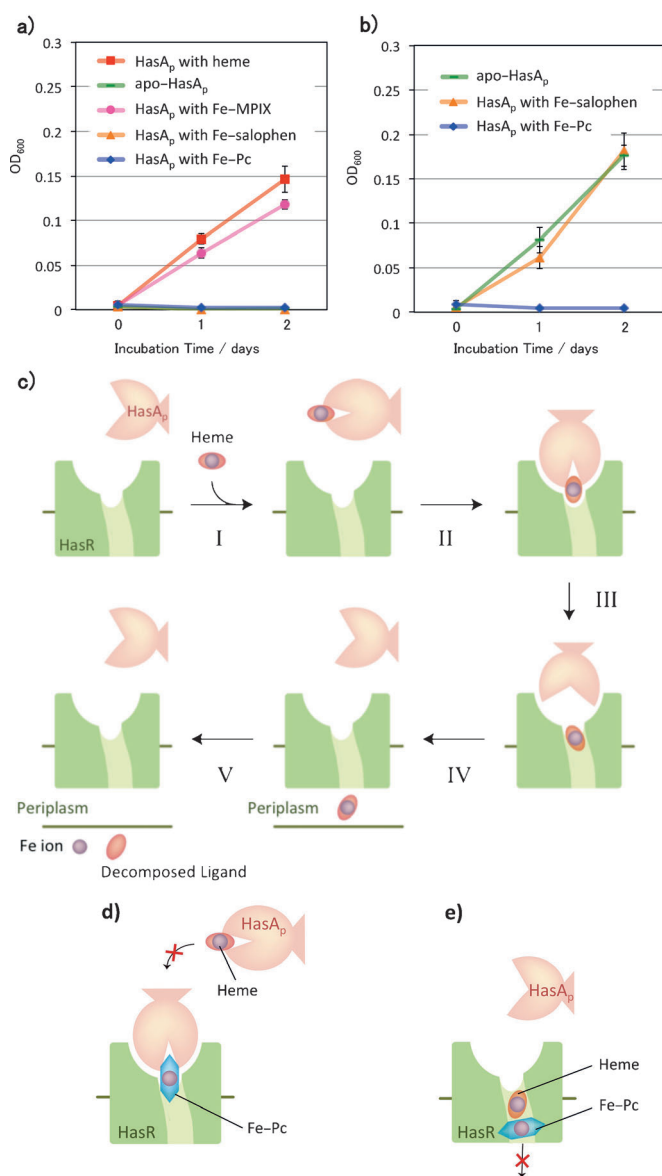


Figure 4. Growth behavior of *P. aeruginosa* in iron-limited medium containing EDTA as a scavenger of iron cations. Cell growth was monitored through optical density at 600 nm (OD₆₀₀). a) Growth of *P. aeruginosa* in the presence of 0.25 μM of heme-bound holo-HasA_p or HasA_p bound to synthetic metal complexes. As a control experiment, apo-HasA_p was also tested (green line). b) The effect of HasA_p bound to synthetic metal complexes on the growth of *P. aeruginosa* in the presence of heme-bound HasA_p. A solution of HasA_p bound to synthetic metal complexes or apo-HasA_p (as a negative control) was added to a suspension of *P. aeruginosa* (the final concentrations were 0.25 μM) provided with 0.5 μM heme-bound holo-HasA_p as an iron source. Error bars represent the standard deviation of at least three measurements. c) Steps of the general heme uptake process by HasA. HasA captures a heme molecule (step I). Holo-HasA then interacts with a specific receptor called HasR (step II) and the heme is transferred from HasA to HasR (step III). The heme passes through HasR and is transported into the periplasm (step IV). The heme is degraded by an enzyme to release an iron cation in the cytoplasm (step V). d) and e) Schematic representations of plausible mechanisms to explain the inhibition of HasA_p-mediated heme uptake by Fe-Pc-bound HasA_p: HasA_p bound to Fe-Pc strongly interacts with HasR and blocks the specific interaction domain of HasR (d); or Fe-Pc is transferred to HasR and blocks the access channel (e).

observed: there was no growth inhibition by Fe-Pc-bound HasA_p either in the absence of EDTA (i.e., in iron-containing medium) or in the presence of free Hemin (Figure S7 and S8). Although further studies on the interaction between HasA_p and HasR, including the transfer of metal complexes from HasA_p to HasR, are required to clarify the mechanism of inhibition by HasA_p bound to Fe-Pc, we propose two possibilities: 1) Fe-Pc-bound HasA_p strongly interacts with HasR and blocks the specific interaction domain of HasR, thereby resulting in a strong inhibition of heme transfer from holo-HasA_p to HasR (step III in Figure 4c, Figure 4d); 2) Fe-Pc is transferred to HasR and blocks the access channel of HasR because of the higher hydrophobicity of Fe-Pc compared to heme (step IV in Figure 4c, Figure 4e). Fe-salophen-bound HasA_p did not inhibit the growth of *P. aeruginosa*, thus suggesting that Fe-salophen bound to HasA could not block the specific interaction domain of HasR or that transferred Fe-salophen was not effective in blocking the access channel of HasR. It is very interesting that the inhibitory effect differs depending on the structure of the metal complexes captured by HasA_p.

In conclusion, we have demonstrated that HasA_p can capture several synthetic metal complexes other than heme. The crystal structures of HasA_p bound to Fe-MPIX, Fe-salophen, and Fe-Pc showed that HasA_p captures these metal complexes in the same fashion as it does heme without any structural perturbation. We also found that HasA_p bound to Fe-Pc strongly inhibited HasA_p-mediated heme acquisition. These findings contribute to the understanding of heme transfer from HasA to HasR. Furthermore, we expect that these observations could lead to the development of a *P. aeruginosa* elimination system that doesn't involve antibiotics.

Received: September 7, 2013

Revised: December 30, 2013

Published online: February 7, 2014

Keywords: growth inhibition · HasA · heme proteins · iron complexes · protein structures

- [1] S. Letoffe, V. Redeker, C. Wandersman, *Mol. Microbiol.* **1998**, 28, 1223–1234.
- [2] a) J. M. Ghigo, S. Letoffe, C. Wandersman, *J. Bacteriol.* **1997**, 179, 3572–3579; b) S. Letoffe, J. M. Ghigo, C. Wandersman, *Proc. Natl. Acad. Sci. USA* **1994**, 91, 9876–9880.
- [3] a) A. Idei, E. Kawai, H. Akatsuka, K. Omori, *J. Bacteriol.* **1999**, 181, 7545–7551; b) S. Letoffe, K. Omori, C. Wandersman, *J. Bacteriol.* **2000**, 182, 4401–4405.
- [4] R. Kumar, S. Lovell, H. Matsumura, K. P. Battaile, P. Moenne-Loccoz, M. Rivera, *Biochemistry* **2013**, 52, 2705–2707.
- [5] S. Létoffé, C. Deniau, N. Wolff, E. Dassa, P. Delepelaire, A. Lecroisey, C. Wandersman, *Mol. Microbiol.* **2001**, 41, 439–450.
- [6] a) S. Krieg, F. Huche, K. Diederichs, N. Izad iPruneyre, A. Lecroisey, C. Wandersman, P. Delepelaire, W. Welte, *Proc. Natl. Acad. Sci. USA* **2009**, 106, 1045–1050; b) N. Izad iPruneyre, F. Huche, G. S. Lukat-Rodgers, A. Lecroisey, R. Gilli, K. R. Rodgers, C. Wandersman, P. Delepelaire, *J. Biol. Chem.* **2006**, 281, 25541–25550.
- [7] P. Arnoux, R. Haser, N. Izadi, A. Lecroisey, M. Delepierre, C. Wandersman, M. Czjzek, *Nat. Struct. Biol.* **1999**, 6, 516–520.

- [8] A. Y. Alontaga, J. C. Rodriguez, E. Schonbrunn, A. Becker, T. Funke, E. T. Yukl, T. Hayashi, J. Stobaugh, P. Moenne-Loccoz, M. Rivera, *Biochemistry* **2009**, *48*, 96–109.
- [9] a) G. Jepkorir, J. C. Rodriguez, H. A. Rui, W. Im, S. Lovell, K. P. Battaile, A. Y. Alontaga, E. T. Yukl, P. Moenne-Loccoz, M. Rivera, *J. Am. Chem. Soc.* **2010**, *132*, 9857–9872; b) N. Wolff, N. Izad iPruneyre, J. Couprie, M. Habeck, J. Linge, W. Rieping, C. Wandersman, M. Nilges, M. Delepierre, A. Lecroisey, *J. Mol. Biol.* **2008**, *376*, 517–525.
- [10] Y. Satake, S. Abe, S. Okazaki, N. Ban, T. Hikage, T. Ueno, H. Nakajima, A. Suzuki, T. Yamane, H. Nishiyama, Y. Watanabe, *Organometallics* **2007**, *26*, 4904–4908.
- [11] M. Ratliff, W. M. Zhu, R. Deshmukh, A. Wilks, I. Stojiljkovic, *J. Bacteriol.* **2001**, *183*, 6394–6403.
-