Siderophore Import

DOI: 10.1002/anie.201005527

Direct Identification of a Siderophore Import Protein Using Synthetic Petrobactin Ligands**

Nikolas Bugdahn, Florian Peuckert, Alexander G. Albrecht, Marcus Miethke, Mohamed A. Marahiel, and Markus Oberthür**

The increase of bacterial resistance against almost all clinically used antibiotics is one of the most pressing public health problems. While existing drugs are becoming less and less effective, only a few truly new antibiotics have found their way to clinical application in the last decades. The inhibition of novel biochemical pathways that are not addressed by currently used antibiotics offers a potentially successful strategy for the development of new drugs able to combat infections caused by resistant bacteria. In this context, the interference with the bacterial uptake of iron promoted by siderophores has become the focus of attention.[1] Siderophores are polar low-molecular-weight molecules with exceptionally high iron-binding affinities that are secreted and reimported by microorganisms through dedicated transport systems. In addition to blocking their biosynthesis, the inhibition of siderophore export and import proteins offers a promising approach for the development of new antibiotics, because siderophore-promoted iron uptake is essential for both the survival and virulence of pathogens.[2] For many bacteria, however, there is only limited information available regarding the export and import systems involved. In addition, siderophore-binding proteins have been discovered so far only indirectly based on homology searches or growth phenotype analysis of mutants.

Herein, we report the first capture of a siderophore-binding protein from cell extracts through a direct interaction with its natural ligand.[3] To this end, we employed affinity chromatography using an immobilized siderophore and subsequent identification of the retained protein by mass spectrometric analysis (Figure 1 A). After overexpression in *Escherichia coli* and purification, biochemical characterization of the recombinant protein was possible. This novel approach allows the selection of specific binding proteins out of a pool of siderophore importers present in cell extracts.

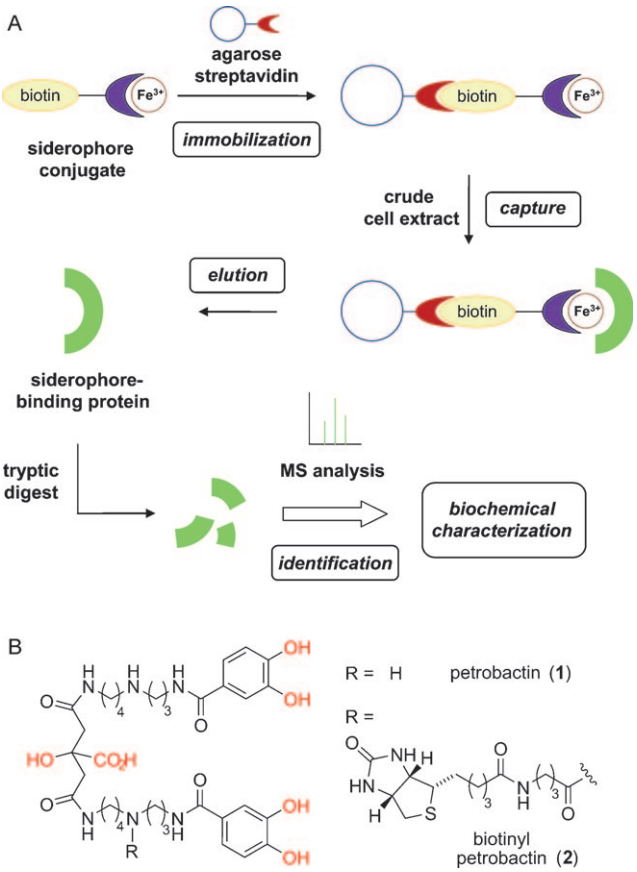


Figure 1. A) Identification of siderophore-binding proteins by affinity chromatography using immobilized siderophores. The retained proteins are identified by MS analysis of fragments obtained by tryptic digest, and are then characterized biochemically. B) Chemical structure of petrobactin (**1**) and biotinyl petrobactin (**2**). The groups that are involved in iron binding are shown in red.

For the capture of siderophore-binding proteins, we selected the siderophore petrobactin (**1**), which most notably is produced by the two pathogens *Bacillus cereus* and *Bacillus anthracis*.[4] For *B. cereus*, two petrobactin transporters have been reported recently based on sequence homologies,[5] whereas genetic studies identified similar petrobactin import systems in *B. anthracis*.[6] For the latter, petrobactin (**1**; Figure 1 B) is a virulence factor, because the second siderophore produced by *B. anthracis*, bacillibactin, is intercepted during an infection by a protein of the innate immune system, siderocalin.[7] Petrobactin (**1**) is therefore essential for

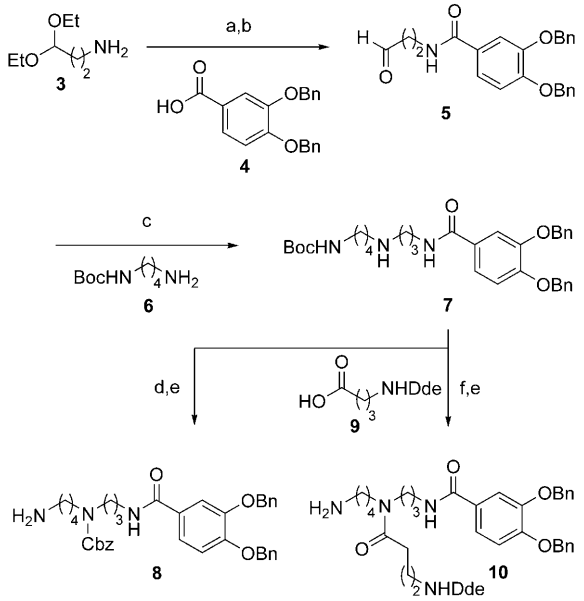
[*] N. Bugdahn,[+] Dr. M. Oberthür
Fachbereich Chemie, Philipps-Universität Marburg
Hans-Meerwein-Strasse, 35032 Marburg (Deutschland)
Fax: (+49) 6421-282-2021
E-mail: oberthuer@chemie.uni-marburg.de
F. Peuckert,[+] A. G. Albrecht, Dr. M. Miethke, Prof. Dr. M. A. Marahiel
Fachbereich Chemie, Philipps-Universität Marburg
Hans-Meerwein-Strasse, 35032 Marburg (Deutschland)
Fax: (+49) 6421-282-2191
E-mail: marahiel@staff.uni-marburg.de
[+] These authors contributed equally to this work.

[**] Financial support from the German Science Foundation (DFG) is gratefully acknowledged. We thank Dr. Uwe Linne and Natalia Fritzler for mass spectrometric analysis and Tanja Ellenberger and Michael Kock (research students) for excellent technical assistance.
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201005527.

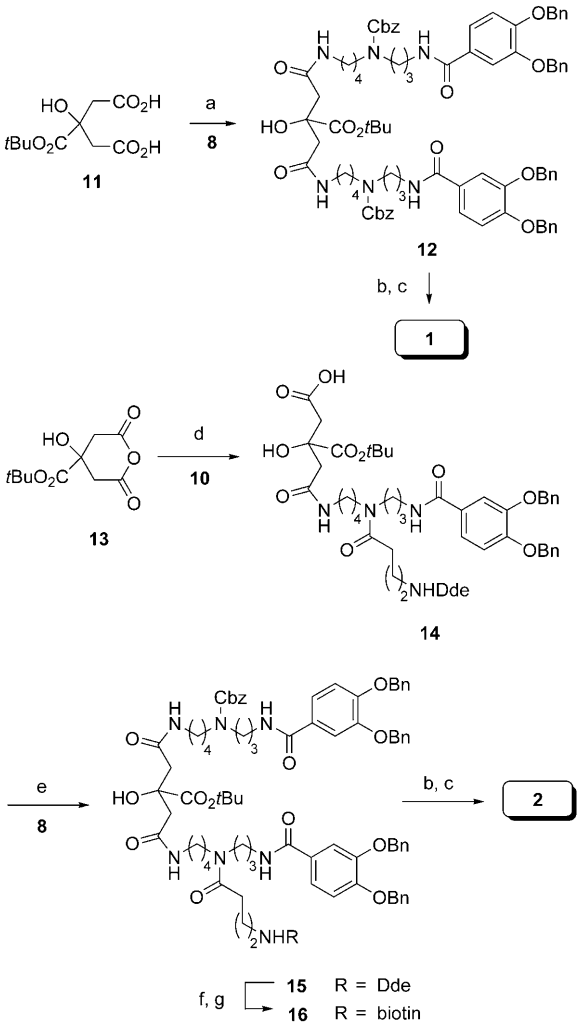
iron acquisition by *B. anthracis* inside a human host, which makes the inhibition of its uptake a potential strategy for treatment of anthrax infections.

Petrobactin (**1**) contains two 3,4-dihydroxybenzoate units attached to a citric acid backbone through spermidine linkers; the two catechol units and the central carboxy and hydroxy groups of the citric acid moiety (red in Figure 1B) act as ligands in the formation of an octahedral iron complex. Because the amino groups of the spermidine units are not directly involved in iron binding, we chose them for the attachment of a biotin tag, such that the siderophore can be immobilized on agarose beads derivatized with streptavidin for affinity chromatography. Accordingly, we synthesized the biotinylated petrobactin **2** (Figure 1B), in which the tag is attached to the central amino group of one of the spermidine side chains by means of a γ-aminobutyric acid spacer. In addition, we also generated sufficient amounts of petrobactin (**1**) for the biochemical characterization of the captured transport protein.

The synthetic route to the required petrobactin spermidine side chain **8** and the modified side chain **10**, which contains the Dde-protected γ-aminobutyric acid linker, started with the commercially available diethyl acetal of 3-aminopropanal, **3**, and the known benzoic acid **4**[8] (Scheme 1). Compared to previously reported syntheses,[8,9] our optimized route to petrobactin side chains is short, operationally simple, and high yielding. In addition, the protection of the central amino group led to improved yields in subsequent coupling reactions (Scheme 2). For the synthesis of petrobactin (**1**), *tert*-butyl citrate **11** was activated as the *N*-hydroxysuccinimide (NHS) ester as described by Phanstiel et al.[8] and subsequently coupled with side-chain amine **8** (87%). Removal of the *tert*-butyl group was accompanied by the formation of small amounts of the known imide side product[8,10] (see the Supporting Information). Hydrogenation and purification of the residue by reversed-phase HPLC then afforded **1** in 30% yield.



Scheme 1. Synthesis of spermidine side chains **8** and **10**. Reagents and conditions: a) **4**, EDC, HOBt, Et_3N, DMF, 92%; b) PPTS, acetone/H_2O, 40°C, 96%; c) **6**, $NaBH(OAc)_3$, Et_3N, MeOH, 71%; d) CbzCl, Et_3N, MeOH, 94%; e) TFA, CH_2Cl_2, 0°C, 95% (**8**); 97% (**10**); f) **9**, EDC, HOBt, Et_3N, DMF, 96%. EDC = 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, HOBt = 1-hydroxybenzotriazole, DMF = *N*,*N*-dimethylformamide, PPTS = pyridinium *para*-toluenesulfonate, Boc = *tert*-butoxycarbonyl, Cbz = benzyloxycarbonyl, Dde = 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl, TFA = trifluoroacetic acid.



Scheme 2. Synthesis of **1** and **2**. Reagents and conditions: a) 1. NHS, DCC, THF; 2. **8**, Et_3N, 1,4-dioxane; 87%; b) AcOH, conc. HCl; c) H_2 (45 bar), Pd–C, EtOH/H_2O, then reversed-phase HPLC purification, 30% (**1**, 2 steps); 26% (**2**, 2 steps); d) **10**, Et_3N, DMF, 84%; e) 1. NHS, DIC, THF; 2. **8**, Et_3N, 1,4-dioxane; 82%; f) N_2H_4, EtOH; g) biotin NHS ester, iPr_2EtN, DMF, 84% (2 steps). DCC = dicyclohexylcarbodiimide, DIC = diisopropylcarbodiimide.

The synthesis of the biotinylated petrobactin **2** commenced with the reaction of *tert*-butyl-protected citric acid anhydride **13** with the linker containing side chain **10**, thus affording monoamide **14** in excellent yield. NHS activation and reaction with petrobactin side chain **8** then led to the fully protected petrobactin derivative **15** (82%). Next, the biotin group was introduced by selective removal of the Dde protecting group using hydrazine and subsequent biotinylation of the liberated amine (84%). Finally, removal of the

remaining protecting groups and HPLC purification afforded biotinylated petrobactin **2**.

When an aqueous solution of biotinyl petrobactin (**2**) was treated with $FeCl_3$ at pH 8, iron complexation was clearly evident because of a strong color change to purple. The formation of the siderophore–iron(III) complex was also confirmed by mass spectrometry. Following incubation of streptavidin-derivatized agarose beads with the iron-loaded siderophore, the purple beads were transferred to a column and equilibrated with PBS buffer. To test whether the immobilized petrobactin derivative **2** is able to capture relevant binding proteins from cell extracts, we chose *Bacillus subtilis* as a model organism. *B. subtilis* does not produce petrobactin (**1**) but is able to scavenge this siderophore from the environment[11] using an import system that was not known at the outset of our investigations. The cell lysate of a *B. subtilis* culture was loaded onto the affinity column. After washing and elution, the obtained fractions were subjected to a tryptic digest, and the fragments were then analyzed by mass spectrometry using the MASCOT software and the MSDB database (see the Supporting Information).[12]

In our initial experiments, a number of proteins were retained by the column. A negative control using a column loaded with unmodified streptavidin–agarose beads, however, showed that most of these proteins bound to the matrix itself. After extensive experimentation, we were able to reduce unspecific binding by pre-incubation of the siderophore-derivatized column with bovine serum albumin. In subsequent experiments, YclQ was the only protein that was retained repeatedly owing to a specific interaction with the petrobactin-derivatized agarose beads (see the Supporting Information). YclQ is an ABC transporter binding protein that is part of the *yclNOPQ* gene cluster. It has only recently been characterized to be the ferric petrobactin-binding protein in *B. subtilis* by the group of Raymond based on sequence homologies,[13] thus confirming the viability of our approach. Because of its function, we propose to rename this gene cluster *fpiBCDA* (fpi: ferric petrobactin import) and, accordingly, rename YclQ FpiA.

To elucidate the role of the identified protein on the genetic level, a *B. subtilis* Δ*fpiA* mutant was created, which is also incapable of producing 2,3-dihydroxybenzoic acid and bacillibactin as endogenous high-affinity chelators as a result of its Δ*dhbC* background.[14] The resulting Δ*dhbC*Δ*fpiA* double mutant and the Δ*dhbC* mutant (as control) were grown under iron limitation, and supplementation of $FeCl_3$, bacillibactin, or petrobactin, respectively (Figure 2). Both mutants showed an increased rate of growth compared to iron limitation when iron(III) or iron-free bacillibactin was added as a result of the uptake of iron by siderophore-independent import systems and the bacillibactin-mediated import of iron present in the culture, respectively.

In contrast to the Δ*dhbC* strain, however, the Δ*dhbC*-Δ*fpiA* double mutant did not grow in the presence of iron-free petrobactin, which indicates that FpiA is the only petrobactin-binding protein of *B. subtilis*. The growth inhibition observed in the case of the Δ*dhbC*Δ*fpiA* mutant can be explained by the action of petrobactin as an antibiotic through the removal of remaining traces of iron from the medium.

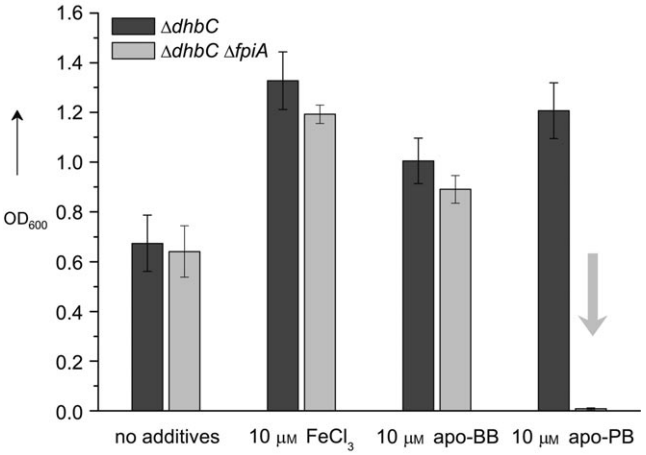


Figure 2. Final OD_{600} values (after 18 h) of Δ*dhbC* and Δ*dhbC*Δ*fpiA* cultures with several additives. The gray arrow indicates the total growth inhibition of the double mutant in the presence of apo-PB. apo-BB = bacillibactin (iron-free), apo-PB = petrobactin (iron-free).

The recombinantly produced protein FpiA was analyzed for binding of the native ligand petrobactin (**1**), its biotinylated analogue **2**, and several other possible ligands by fluorescence spectroscopy (see the Supporting Information). The FpiA binding constant obtained for petrobactin (51 nM) is in good accordance with previously published data.[13] In comparison, the ferric complex of the biotinylated petrobactin **2** is bound almost three orders of magnitude more weakly by FpiA. Nevertheless, the capture of FpiA clearly shows that this decreased binding affinity is still sufficient enough for the successful retention of binding proteins.[15]

The binding stoichiometry of FpiA and the native iron-loaded ligand **1** is rather unusual. In our fluorescence titration experiments we determined a protein to ligand ratio of 1:4. To corroborate the fluorescence measurements, we further examined the protein–siderophore interaction using ligand-dependent melting-point analysis.[16] The greatest increase in the melting point because of the ligand-induced stabilization of the complex was again achieved with a protein to ligand ratio of 1:4 or higher (Figure 3). The same stoichiometry was

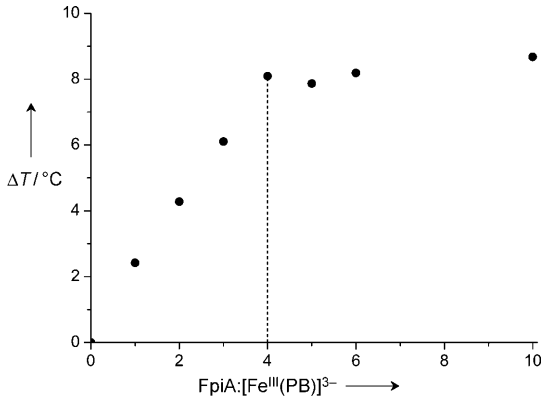


Figure 3. Petrobactin-dependent thermal stabilization of FpiA. The melting points were determined by CD spectroscopy, and maximal stabilization (Δ*T*) was observed at a protein/ligand ratio of 1:4 or higher.

recently obtained for the petrobactin-binding proteins FatB and FpuA from *B. cereus*.[5] Nevertheless, since most siderophore-binding proteins interact with their ligand in a 1:1 ratio, further analysis of the FpiA–petrobactin interaction is warranted.

In conclusion, we have demonstrated that the biotinylated petrobactin derivative **2** described here is a valuable tool for the direct identification of siderophore-binding proteins using affinity chromatography. The robustness of our method is shown by the successful retention and identification of the membrane-bound protein FpiA, the principal petrobactin-binding protein in *B. subtilis*. This novel approach currently extends to the isolation of petrobactin-binding proteins from pathogenic bacteria, for example, *B. anthracis*. Importantly, it should be useful for the capture of binding proteins that have no homologies to known siderophore transporters. The identification and subsequent biochemical and structural characterization of such transport proteins represent first steps towards the development of antibiotics that interfere with bacterial iron transport.

Received: September 3, 2010
Published online: November 29, 2010

Keywords: affinity chromatography · immobilization · petrobactin · siderophore import proteins · siderophores

[1] a) M. Miethke, M. A. Marahiel, *Microbiol. Mol. Biol. Rev.* **2007**, *71*, 413 – 415; b) K. D. Krewulak, H. J. Vogel, *Biochim. Biophys. Acta Biomembr.* **2008**, *1778*, 1781 – 1804; c) R. C. Hider, X. Kong, *Nat. Prod. Rep.* **2010**, *27*, 637 – 657.

[2] The exploitation of siderophore uptake systems to deliver siderophore conjugates that contain antibiotic moieties (sideromycins or "Trojan-horse antibiotics") into bacterial cells has been studied more extensively than the inhibition of siderophore uptake (see Ref. [1] for an overview).

[3] Using a related methodology, the siderophore pyoverdin, immobilized on gold-plated glass chips, has recently been used to capture and identify the bacterium *Pseudomonas aeruginosa* (but no specific importer): D. D. Doorneweerd, W. A. Henne, R. G. Reifenberger, P. S. Low, *Langmuir* **2010**, *26*, 15424 – 15429.

[4] a) M. K. Wilson, R. J. Abergel, K. N. Raymond, J. E. Arceneaux, B. R. Byers, *Biochem. Biophys. Res. Commun.* **2006**, *348*, 320 – 325; b) K. Hotta, C. Y. Kim, D. T. Fox, A. T. Koppisch, *Microbiology* **2010**, *156*, 1918 – 1925; c) A. T. Koppisch, C. C. Browder, A. L. Moe, J. T. Shelley, B. A. Kinkel, L. E. Hersman, S. Iyer, C. E. Ruggiero, *BioMetals* **2005**, *18*, 577 – 585.

[5] A. M. Zawadzka, R. J. Abergel, R. Nichiporuk, U. N. Andersen, K. N. Raymond, *Biochemistry* **2009**, *48*, 3645 – 3657.

[6] P. E. Carlson, Jr., S. D. Dixon, B. K. Janes, K. A. Carr, T. D. Nusca, E. C. Anderson, S. E. Keene, D. H. Sherman, P. C. Hanna, *Mol. Microbiol.* **2010**, *75*, 900 – 909.

[7] R. J. Abergel, M. K. Wilson, J. E. Arceneaux, T. M. Hoette, R. K. Strong, B. R. Byers, K. N. Raymond, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 18499 – 18503.

[8] R. A. Gardner, R. Kinkade, C. Wang, O. Phanstiel IV, *J. Org. Chem.* **2004**, *69*, 3530 – 3537.

[9] R. J. Bergeron, G. Huang, R. E. Smith, N. Bharti, J. S. McManis, A. Butler, *Tetrahedron* **2003**, *59*, 2007 – 2014.

[10] The propensity of citric acid based siderophores to form imides has long been known: B. H. Lee, M. J. Miller, *J. Org. Chem.* **1983**, *48*, 24 – 31, and references therein.

[11] R. J. Abergel, A. M. Zawadzka, K. N. Raymond, *J. Am. Chem. Soc.* **2008**, *130*, 2124 – 2125.

[12] D. N. Perkins, D. J. Pappin, D. M. Creasy, J. S. Cottrell, *Electrophoresis* **1999**, *20*, 3551 – 3567.

[13] A. M. Zawadzka, Y. Kim, N. Maltseva, R. Nichiporuk, Y. Fan, A. Joachimiak, K. N. Raymond, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 21854 – 21859.

[14] M. Miethke, O. Klotz, U. Linne, J. J. May, C. L. Beckering, M. A. Marahiel, *Mol. Microbiol.* **2006**, *61*, 1413 – 1427.

[15] FpiA could show higher affinity for biotinyl petrobactin **2** on a solid support because of a multivalent binding mode.

[16] F. Peuckert, M. Miethke, A. G. Albrecht, L.-O. Essen, M. A. Marahiel, *Angew. Chem.* **2009**, *121*, 8066 – 8069; *Angew. Chem. Int. Ed.* **2009**, *48*, 7924 – 7927.

