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Nitro versus Hydroxamate in Siderophores of Pathogenic Bacteria: Effect of Missing Hydroxylamine Protection in Malleobactin Biosynthesis**

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Siderophores play a major role as virulence factors of pathogenic bacteria.[1] Known as the strongest Fe^{3+}-binding agents, these structurally diverse compounds are used to scavenge scarcely soluble iron from the human or animal hosts. Strikingly, infamous human pathogenic bacteria like *Yersinia pestis*, the causative agent of devastating black death epidemics, completely lose their virulence in the absence of a siderophore.[2] Thus, knowledge of a pathogen's siderophore structures and of the corresponding biosynthetic machineries is considered a prerequisite for new therapeutic approaches such as targeting pathogenicity factors and Trojan horse strategies using siderophore–drug conjugates.[3] Over the past two decades, much research has been devoted to elucidating the siderophores of *Burkholderia mallei* and *Burkholderia pseudomallei*,[4–7] the causative agents of the infectious diseases glanders[8] and melioidosis.[9] Infections with these β-proteobacteria are often lethal, even with the best treatment available.[10] Both species have thus been categorized as potential biological warfare agents, and indeed *B. mallei* has been abused in World War I to kill enemy horses and mules.[11,12] Although it has long been known that *B. pseudomallei* and *B. mallei* produce two types of siderophores, pyochelin (**1** and 2″-*epi*-**1**, Figure 1) and malleobactin (**2**),[4] surprisingly, the structure of the latter has remained obscure. It was only a matter of speculation that malleobactin could be related to the ornibactins (**3**–**5**, Scheme 1), hydroxamate siderophores produced by the *Burkholderia cepacia* complex (Bcc).[5,6] Here we disclose the unusual structure and absolute configuration of malleobactin, the siderophore of the human pathogenic *B. mallei* family and reveal the biogenetic origin of an unprecedented aliphatic nitro amino acid.

To gain first insights into the structural deviations of the encoded siderophores, we compared the gene loci for ornibactin and malleobactin biosynthesis.[6,13] Both types of gene clusters share genes for a tetramodular nonribosomal peptide synthetase (NRPS) and accessory enzymes. Furthermore, genes for putative amino acid tailoring enzymes (ornithine monooxygenase,[14] aspartic acid β-hydroxylase,[15] *N*-formyltransferase[16]), siderophore receptors and transporters are present in both types of gene clusters. However, we noted that the gene loci in bacteria belonging to the *B. mallei* family harbor an additional gene for a hypothetical protein (*mbaM*), but lack orthologues of *orbK* and *orbL* (Figure 1 A, yellow open reading frames (orfs)). The latter genes code for acyltransferases, and although their biochemical function has not yet been studied, one may assume that they are required for loading acyl units onto the N-terminal ornithine residue.[13] We concluded that ornibactins and malleobactin differ in their substitution patterns.

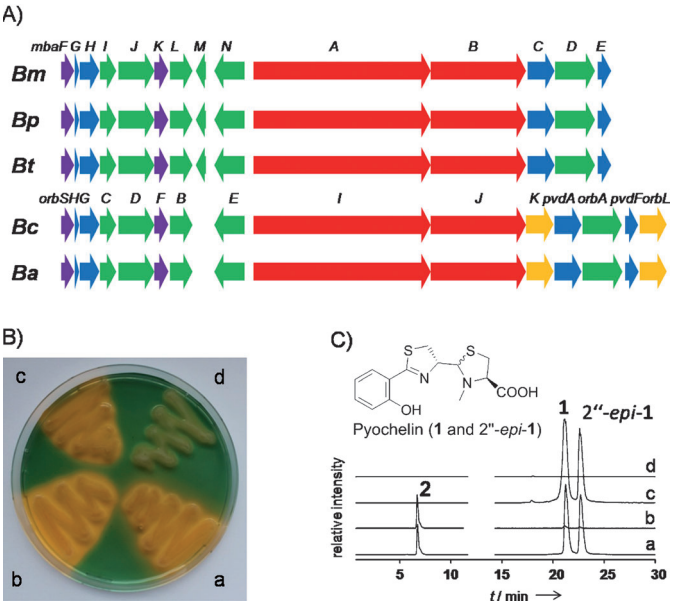

Figure 1. Comparison of malleobactin and ornibactin biosynthesis gene clusters (*mba* and *orb*, respectively) of *Burkholderia* spp., and analysis of siderophore production of *B. thailandensis*. A) Malleobactin biosynthesis gene clusters: *Bm*: *B. mallei*; *Bp*: *B. pseudomallei*; *Bt*: *B. thailandensis*. Ornibactin biosynthesis gene clusters: *Bc*: *B. cenocepacia*; *Ba*: *B. ambifaria*. Deduced functions of *mba* biosynthesis genes, see Table S2 in the Supporting Information. *pvd* genes refer to orthologues from the pyoverdin biosynthesis gene cluster in *Pseudomonas* spp. B) CAS agar plate assay of *B. thailandensis* wild type (a), Δ*pchE* mutant (b, *pch* = pyochelin biosynthesis gene cluster), Δ*mbaA* mutant (c), Δ*pchE*Δ*mbaA* mutant (d). C) LC–MS profiles monitoring malleobactin (**2**, *m*/*z* 637) and pyochelin (**1** and 2″-*epi*-**1**, *m*/*z* 325) production of mutant strains (as in (B)).

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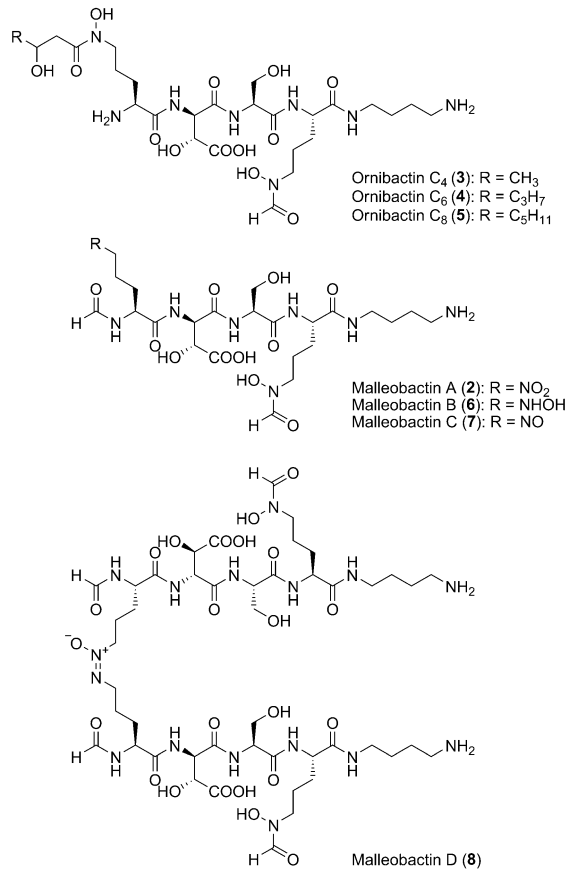
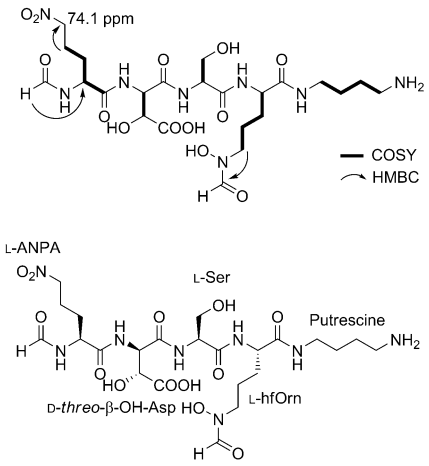


Scheme 1. Structures of ornibactins (**3**–**5**) and malleobactin A (**2**), B (**6**), C (**7**), and D (**8**).

For functional analyses of the gene cluster and to elucidate the structure of the encoded siderophore, we chose *Burkholderia thailandensis*, the least virulent member of the *B. mallei* complex as a model.[17] To reveal the potential of *B. thailandensis* to produce *B. mallei*-type siderophores we analyzed metabolic profiles of the wild type and engineered strains deficient in functional pyochelin and malleobactin biosynthesis gene clusters. Therefore, we specifically disrupted genes coding for the putative pyochelin and malleobactin NRPS modules by homologous recombination. By means of a CAS (chrome azurol S) assay to detect Fe^{3+} binding capacities of secreted compounds we corroborated that both gene clusters are sufficient and necessary for siderophore activity in *B. thailandensis* (Figure 1 B). LC–MS monitoring of cultures grown under iron limitation proved the correct assignment of the pyochelin and malleobactin biosynthesis gene clusters (Figure 1 C). The highly polar compound **2** with a molecular weight of 636 Da, which corresponds to the mystery siderophore reported earlier,[6] is only detectable in the wild type, but not in the mutant strain. As we detected several putative congeners with similar retention time and molecular weight in the crude extracts, we named the main compound malleobactin A. To elucidate the structure of malleobactin A (**2**), crude extracts of *B. thailandensis* cultures grown in iron-deficient media were subjected to size-exclusion chromatography.[6] After multiple rounds of preparative HPLC we obtained a sample of pure malleobactin A and solved its structure by extensive 2D NMR spectroscopy (Scheme 2).

In D_2O five spin systems were identified by using COSY that corroborated the predicted relationship with the ornibactins. Four spin systems readily identified putrescine, N^{δ}-hydroxy-N^{δ}-formylornithine (hfOrn), serine, and β-hydroxy-aspartic acid (OH-Asp) as peptide building blocks. The remaining spin system, however, did not fit to the N-terminal ornibactin residue, N^{δ}-hydroxy-N^{δ}-acylornithine (haOrn). A proton resonating at 4.37 ppm, which was assigned as the α-proton of the remaining amino acid, was used as the starting point of this spin system. From $^4J_{HH}$ and HMBC correlations we concluded that a formyl group at $\delta_H = 8.01$ ppm is connected to this amino group and that this amino acid is located at the N terminus. Continuing from the CH^{α} group, two neighboring methylene groups ($\delta_H = 1.73$ and 1.91 ppm, respectively) were detected. Whereas no additional coupling protons could be detected in the COSY spectrum, the HMBC spectrum showed a correlation of the outer methylene group to a carbon resonating at 74.1 ppm, which could not be detected in the ^{13}C spectrum in D_2O. MS measurement of the sample incubated in D_2O showed a mass shift of 2 Da, indicating proton–deuterium exchange owing to the presence of a highly acidic functional group. Since the resulting CD_2 group corresponds to a quaternary carbon atom split into a 1:2:3:2:1 multiplet, it could not be detected in the ^{13}C spectrum. Indeed, further NMR measurements in [D_6]DMSO revealed a C-H acidic methylene group ($\delta_H = 4.55$ ppm, $\delta_C =$ 74.9 ppm). In light of the unusual chemical shifts, the high acidity, the molecular weight, and the biosynthetic connection to the ornibactins, it was evident that the remaining amino acid features a terminal nitro group. This interpretation was backed up by the IR spectrum of **1**, which shows bands for NO valence vibrations at 1530 and 1359 cm^{-1}.[18] Thus, the missing amino acid represents 2-amino-5-nitropentanoic acid (ANPA). Notably, this unusual nitro amino acid has only been known as a synthetic compound and is here presented for the first time as part of a natural product. Finally, we



Scheme 2. Structure elucidation of malleobactin A (**2**). Key COSY and HMBC correlations in D_2O, and results from amino acid analysis.

assigned the sequence of the amino acid residues by HMBC correlations in [D_6]DMSO and MS/MS fragmentation patterns (Figures S4 and S5 in the Supporting Information).

The relative configuration of OH-Asp was determined to be *threo* by *J*-based analysis ($^3J_{HH} = 2.8$ Hz, $^2J_{H^{\alpha}C^{\beta}} = -3$ Hz, $^2J_{H^{\beta}C^{\alpha}} = -2$ Hz).[19] The absolute configuration of **2** was elucidated using Marfey's method. Thus, we found that **2** is composed of L-serine, D-*threo*-β-hydroxyaspartic acid and L-hfOrn. ANPA was synthesized as a reference compound as reported.[20] However, to our surprise neither L- nor D-ANPA could be detected in the hydrolysate, but L-glutamate, which proved to be a degradation of product of L-ANPA. We rationalized this unexpected finding by a nitro–nitrite rearrangement, loss of NO, and subsequent oxidation with in situ formed nitrosyl chloride (Figure S6 in the Supporting Information).[21] Thus, we solved the absolute configuration of all residues and showed that malleobactin A differs from ornibactin in an unusual α-formylated L-ANPA in lieu of a L-haOrn unit.

Aliphatic nitro compounds such as ANPA are extremely scarce in nature, and only little is known about their biosynthesis.[22] The most plausible mechanism involves a full 6-electron oxidation of the corresponding amine. Whereas it has been proposed that during the biosynthesis of nitrosugars[23] such as kijanose a single enzyme would catalyze the complete oxidation to the nitro group,[24] in vitro enzyme assays only showed the production of the corresponding hydroxylamine.[25] For evernitrose it has been demonstrated that a nitrososynthase catalyzes the sequential 4-electron oxidation to the nitroso sugar, which is oxidized spontaneously to the nitro compound.[26,27] Only for aromatic nitro groups, an enzymatic full 6-electron oxidation of the corresponding amine has been reported.[28]

To gain insights into the biosynthesis of the unusual nitro-substituted siderophore **2** we examined the metabolic profile of *B. thailandensis* more closely. Indeed, in crude extracts we detected compounds with MS and MS/MS data corresponding to the hydroxylamine **6** and the nitroso derivative **7**, which we termed malleobactin B and C (Scheme 1). Isolation and full characterization of **6** confirmed the identity of the hydroxylamine moiety. We also detected a compound (**8**) with a molecular weight of 1224 Da, suggesting that it could result from a fusion of two malleobactin units. In fact, MS/MS as well as NMR data of a pure sample of **8** revealed that it represents the azoxy-linked dimer malleobactin D. As we have shown earlier, aromatic azoxy natural products may be formed from hydroxylamine and nitroso intermediates during the stepwise enzymatic oxidation of aromatic amino groups.[28] In light of these findings, we concluded that a sequential 6-electron transfer leads to the aliphatic nitro group of malleobactin.

To shed more light on the biosynthesis of the nitro group we re-examined the *mba* gene cluster. Yet, we gathered only one candidate oxygenase gene, *mbaC*, which could be involved in nitro group formation. The deduced gene product, MbaC, belongs to the well-studied class of flavin-dependent ornithine monooxygenases involved in N-hydroxylation.[14] Interestingly, neither phylogenetic analyses (Figure 2A) including known ornithine and lysine monooxygenases nor comparison of MbaC with the well-characterized ornithine monooxygenase PvdA[29] by means of homology modeling and sequence alignment (Figures S12 and S13 in the Supporting Information) revealed any differences that could explain over-oxidation of the substrate. Thus, to test the function of MbaC, we heterologously produced the enzyme in *E. coli* and performed an in vitro assay[30] using ornithine as a substrate. In full agreement with the predicted function of MbaC, *N*-hydroxyornithine represented the predominant oxygenation product (Figure 2B, trace b). Furthermore, we detected small amounts of ANPA as well as traces of the putative nitroso intermediate and the azoxide shunt product. The results from the MbaC assay indicated that the hydroxylamine **6** is the actual product of the *mba* NRPS (Figure 2D). Derivatives of higher oxidation states are more likely formed by autoxidation. This model would be in full accord with the reported reactivity of aliphatic hydroxylamines.[31] To test this hypothesis we incubated the hydroxylamine malleobactin B (**6**) under physiological conditions. Within several hours, **6** was readily transformed nonenzymatically into malleobactin C (**7**), malleobactin A (**2**), and the side product malleobactin D (**8**; Figure 2C). We also observed the formation of a yet uncharacterized isomer of malleobactin B, which can also be detected in small amounts in culture extracts. As to the timing of N-formylation, nonformylated precursors could not be detected that would provide evidence for a post-NRPS tailoring reaction. However, the adenylation domain A^1 contains a conserved aspartate residue[32] that is required for interactions with free α-amino groups. The biosynthesis is completed by nucleophilic release of the nascent peptide chain with putrescine, catalyzed by condensation domain C^4 in lieu of a thioesterase domain.[33]

From these genetic, biochemical, and chemical data we concluded that N-acylation in hydroxamate siderophore pathways has not only evolved to provide a bidentate ligand for ferric iron coordination, but is also a prerequisite to protect the *N*-hydroxy group from further oxidation. Interestingly, diverse natural product structures suggest that similar scenarios take place in various other biosynthetic pathways. For example, the siderophores IC202B and IC202C[34] could originate from a truncated desferrioxamine pathway that lacks acylation of the terminal hydroxylamine (Figure S16 in the Supporting Information). On the other hand, in the absence of *N*-acyl groups, N-hydroxylation may set the stage for intriguing downstream reactions. For example, it has been implicated that the piperazic acid like amino acids of the nonribosomal peptides kutznerides are derived from *N*-hydroxyornithine.[35] Our findings support a model according to which dehydropiperazic acid is formed by spontaneous oxidation to the nitroso intermediate followed by cyclization to a stable six-membered ring (Figure S17 in the Supporting Information). Nonetheless, to our knowledge the formation of nitroso and nitro derivatives has not been reported to date in the context of in vitro assays with ornithine and lysine N-monooxygenases.[35,36]

In conclusion, we have elucidated the structure of malleobactin A, the long-sought-after siderophore of animal and human pathogenic bacteria of the *B. mallei* complex. Through gene cluster analyses and targeted knock-outs, we

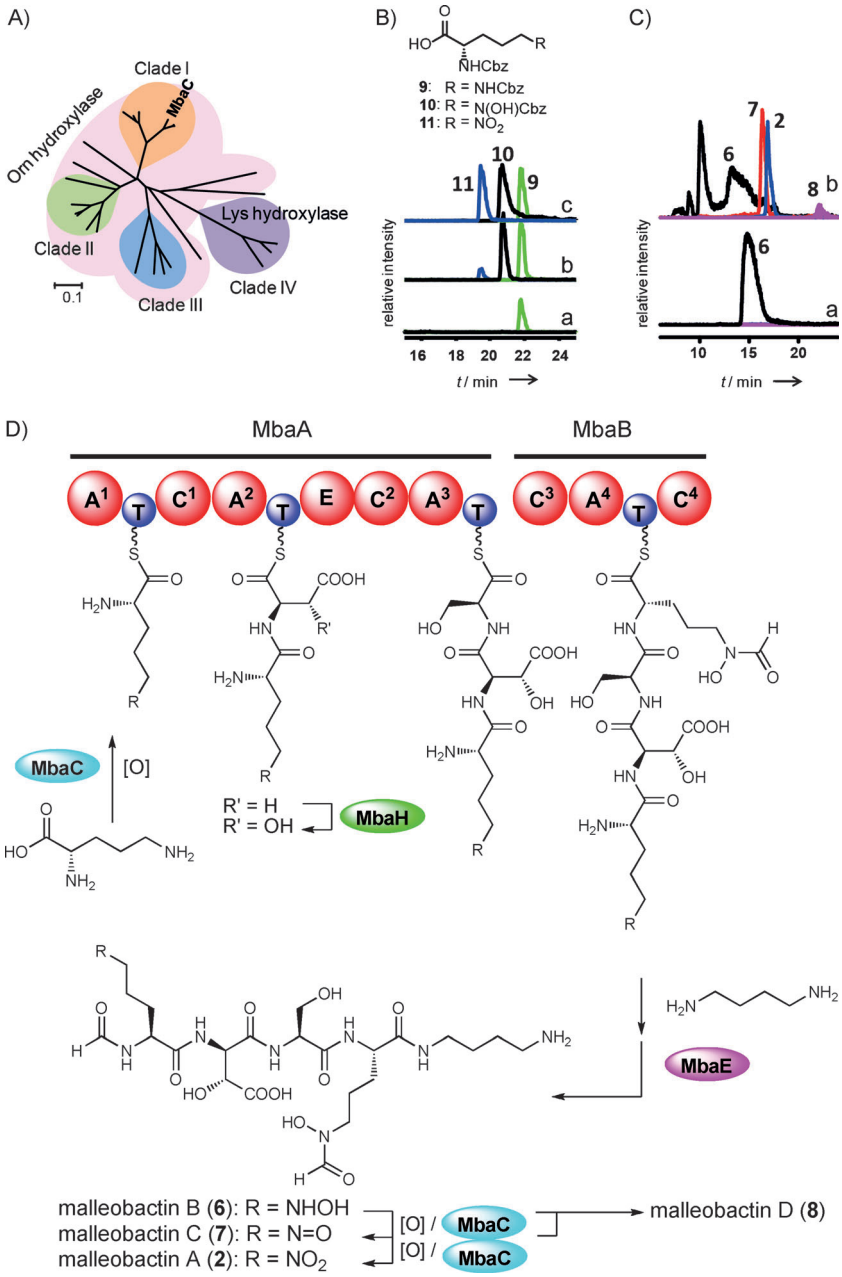


Figure 2. Bioinformatics, biochemical, and chemical analyses of the biosynthesis of malleobactins A–D. A) Cladogram of known ornithine and lysine monooxygenases. Clade I: PvdA orthologues from *Burkholderia* spp. Clade II: PvdA orthologues from the pyoverdin pathway in *Pseudomonas* spp. Clade III: SidA orthologues from siderophore pathways in *Aspergillus fumigatus*. Clade IV: MbtG orthologues from the mycobactin pathway in *Mycobacterium* spp. The scale bar indicates amino acid substitutions per site. B) LC–MS profiles for MbaC-catalyzed N-hydroxylation activity assay (negative ion mode). The traces show benzyloxycarbonyl (Cbz)-derivatized products of heat-denatured MbaC + ornithine (a), MbaC + ornithine (b), reference compounds ornithine (**9**), *N*-hydroxyornithine (**10**), ANPA (**11**) (c). C) LC–MS profiles showing nonenzymatic degradation of hydroxylamine **6** (*m*/*z* 623) to nitroso, azoxy, and nitro derivatives **7** (*m*/*z* 621), **8** (*m*/*z* 613), and **2** (*m*/*z* 637), respectively, in minimal medium at 30 °C after 0 h (a) and after 48 h (b) (positive ion mode). D) Biosynthetic model. A, adenylation; T, thiolation; C, condensation; E: epimerase; TE, thioesterase; MbaC: ornithine monooxygenase; MbaE: formyltransferase; MbaH: hydroxylase.

have unequivocally correlated siderophore activity to the *mba* gene cluster in *B. thailandensis*, the less-virulent model organism of the *B. mallei* family. In addition to disclosing an extraordinary siderophore variant featuring a nitro group, we report the unusual ω-nitro amino acid ANPA for the first time as part of a natural product. The biosynthesis of the aliphatic nitro group was inferred from in vitro studies with the N-hydroxylase MbaC and the detection of the hydroxylamine and nitroso congeners malleobactin B and C, respectively, as well as the azoxy shunt product malleobactin D. To our knowledge, natural products containing *N*-hydroxyornithine without acylation are unprecedented. Moreover, we demonstrate that the unprotected hydroxylamine malleobactin B oxidizes spontaneously to give the nitroso and nitro derivatives. Our in vitro studies with ornithine and MbaC fully support this new scheme for aliphatic nitro group formation. A prerequisite for generating the reactive precursor is the lack of genes coding for dedicated *N*-acyltransferases (such as OrbK and OrbL from the ornibactin biosynthesis gene cluster). These results imply that hydroxamate formation in other siderophore pathways serves as a natural functional group protection to prevent overoxidation. Overall, our study not only unveils the chemical structure of a virulence factor of an important pathogenic bacterium, but also showcases a novel route towards rare aliphatic nitro compounds.

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