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Evolution of Siderophore Pathways in Human Pathogenic Bacteria

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Supporting Information

ABSTRACT: Ornibactin and malleobactin are hydroxamate siderophores employed by human pathogenic bacteria belonging to the genus *Burkholderia*. Similarities in their structures and corresponding biosynthesis gene clusters strongly suggest an evolutionary relationship. Through gene coexpression and targeted gene manipulations, the malleobactin pathway was successfully morphed into an ornibactin assembly line. Such an evolutionary-guided approach has been unprecedented for nonribosomal peptide synthetases. Furthermore, the timing of amino acid acylation before peptide assembly, the absolute configuration of the ornibactin side chain, and the function of the acyl transferase were elucidated. Beyond providing a proof of principle for the rational design of siderophore pathways, a compelling model for the evolution of virulence traits is presented.

Incurable bacterial infections cause a constant threat to humans. The genus *Burkholderia* comprises several species whose pathogenicity greatly impacts our society, foremost *Burkholderia cepacia*[1] and *Burkholderia mallei*.[2] While *B. cepacia* and related species cause severe infections in patients suffering from cystic fibrosis,[3] *B. mallei* and the closely related strain *Burkholderia pseudomallei* have even been classified as potential biological warfare agents.[4] The mortality rate for melioidosis, the infectious disease caused by *B. pseudomallei*, ranges between 16 and 44%, regardless of the use of antibiotics.[5] For survival in animal or human tissue, pathogenic bacteria employ siderophores that withdraw iron from the host. In the search for small molecule virulence factors it was revealed that *B. cepacia*-like strains are capable of producing the hydroxamate siderophore ornibactin,[6,7] whereas *B. mallei*-like strains synthesize a highly similar compound named malleobactin (Figure 1).[8,9] The respective biosynthesis gene clusters show high homology, and the encoded nonribosomal peptide synthetases (NRPS)[10] for siderophore assembly are almost identical. However, two genes *orbK* and *orbL*, putatively coding for acyltransferases,[7] are absent in the malleobactin biosynthesis gene cluster (Figure 1). While it is well-known that biosynthetic gene clusters often diverge by loss or gain of genes or by mutations that lead to loss or gain of function,[11,12] the precise evolutionary course usually remains elusive. Nonetheless, such knowledge is of utmost importance to understand the genesis of small molecule virulence factors. Here we present our success in altering the biosynthetic capabilities of a malleobactin-producing strain to produce ornibactin by genetic manipulation to gain deeper insight into the divergence of both biosynthetic pathways. Notably, such evolutionary-guided engineering approaches[13] have been unprecedented for nonribosomal peptides. As both siderophores are virulence factors of human pathogenic bacteria, our study provides valuable insights into the evolution of pathogenicity traits.

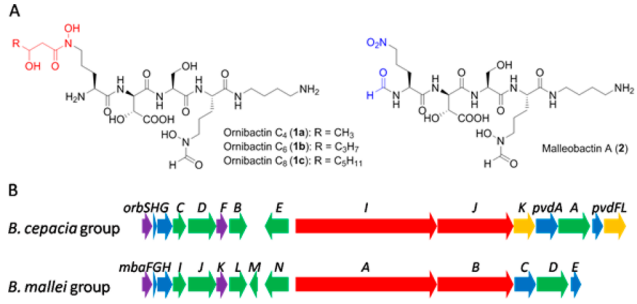

Figure 1. Comparison of ornibactin and malleobactin at structural and genetic levels. (A) Structures of ornibactins from *B. cepacia*-like strains and malleobactin from *B. mallei*-like strains. (B) Biosynthetic gene clusters for ornibactin and malleobactin production.

The *orb* and *mba* gene loci differ in the putative acyltransferase genes *orbK* and *orbL* (Figure S1). Thus, to morph the *mba* biosynthetic machinery into an *orb* assembly line, we initially sought to supplement the *mba* gene cluster with *orbK* and *orbL*. To avoid the use of human pathogenic bacteria we used the less virulent, commonly employed model strain *Burkholderia thailandensis* E264,[14] that is highly similar to *B. mallei* and known as a malleobactin producer.[9] In short, *orbK* and *orbL* were cloned either as single genes or consecutively into an expression vector (pJB861), and the resulting constructs were transferred into *B. thailandensis* by conjugation. Crude extracts of the resulting bacterial mutant cultures were analyzed by LCMS to monitor ornibactin production. Regardless of induction times and constructs, however, ornibactin could not be detected in the mutant broths (Figure 2A, trace vi). On closer inspection we noted that coexpression of *orbL* led to the production of a new compound (**3**) with a mass of 290 Da. MS and NMR data revealed that **3** represents N^{δ}-hydroxy-N^{δ}-(3-hydroxyoctanoyl)-ornithine (C_8-haOrn) (Figure 2C), the modified amino acid that is supposedly activated by the A_1 domain and loaded onto the ornibactin NRPS.[7] By HR-LCMS we also detected analogues of **3** bearing C_6 and C_4 side chains. The successful amino acid acylation

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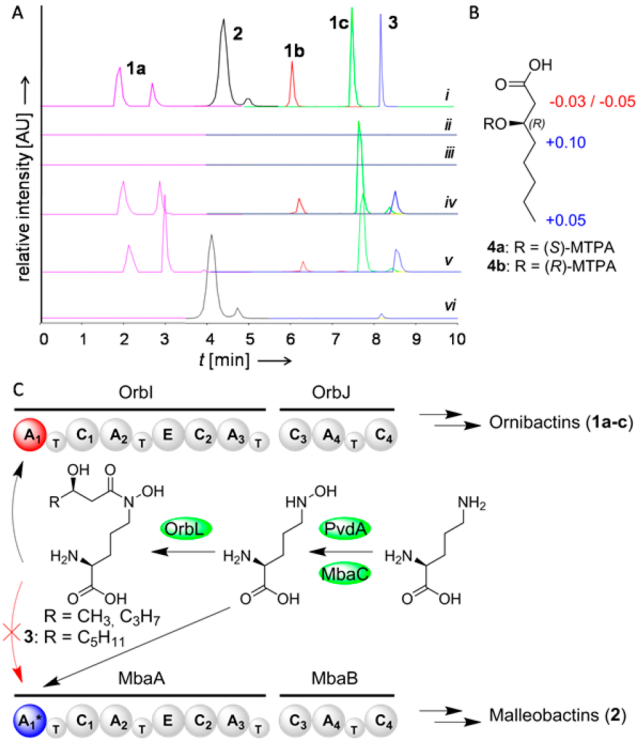

Figure 2. Production of ornibactins (**1a**–**1c**), its building block **3** and malleobactin A (**2**) in *B. thailandensis* wild-type and mutant strains. (A) EIC profiles of standards (i) compared to *B. thailandensis* Δ*mbaA*::*orbI* without additional plasmids (ii), with pJB861-orbK (iii), pJB861-orbL, (iv) and pJB861-orbKL (v). In comparison, wild-type *B. thailandensis* with pJB861-orbKL is shown (vi). The intensity of the ornibactin-C4 (**1a**) peak has been increased 200-fold. (B) Mosher analysis of 3-hydroxyoctanoic acid (**4**) derived from ornibactin-C_8 (**1c**). $\Delta\delta^{SR}$ values are given in ppm. (C) Biosynthetic scheme comparing ornibactin and malleobactin assembly lines showing the differing selection of amino acids at the first adenylation domain.

clearly showed that *orbL* was correctly expressed and functional. Yet, since only the coexpression of *orbL* in a malleobactin-producing strain did not restore ornibactin biosynthesis, it became evident that the ornibactin and malleobactin biosynthesis gene clusters have a higher degree of divergence than expected.

The fact that C_8-haOrn (**3**) was not incorporated by the NRPS suggested that the specificity of the adenylation domain A_1 in the malleobactin synthetase differs from its counterpart in the ornibactin synthetase. Although the A_1 domains show an amino acid sequence identity of 67% and a similarity of 75%, their 8-amino-acid specificity codes[15] clearly differ (Figure S4, S5). For the malleobactin A_1 domain the code is DVETLGGI, whereas the ornibactin A_1 domain code is DAEAAGGI. For comparison, it should be highlighted that the A_4 code (DGEYTGGI) for selection of the highly similar amino acid N^{δ}-hydroxy-N^{δ}-formyl-ornithine (hfOrn) is identical for OrbJ and MbaB. It is interesting to note that A_1 is the only adenylation domain whose specificity code is not completely conserved in both assembly lines. This is in perfect concordance with the fact that structural differences of the two siderophores are only found at the N-terminus of the peptide chain.

Since the different adenylation domain specificity could prevent ornibactin formation, we next addressed this potential bottleneck. By homologous recombination we successfully replaced the *mbaA* sequence coding for the A_1 domain by the corresponding sequence of *orbI*, yielding the mutant strain *B. thailandensis* Δ*mbaA*::*orbI* (see Supporting Information). The mutant strain expressing *orbL* produces all ornibactins (Figure 2A, trace iv). Surprisingly, OrbL was sufficient to generate all ornibactin analogues **1a**–**1c**, whereas for OrbK no *in vivo* activity could be deduced from LCMS/MS data (Figure 2A, trace iii). Since coexpression of *orbK* and *orbL* did not change the metabolic profiles we could exclude a cooperative action of OrbK and OrbL (Figure 2A, trace v). Additional phylogenetic analysis of ornithine-acylating enzymes (Figure S6) and a dotplot self-comparison of the corresponding nucleotide sequences (Figure S7) suggest that OrbK has either a different, yet unknown, or even no function at all. In fact, there are similar scenarios known in which only one out of two homologues is involved in the biosynthetic pathway.[16]

These data clearly show that OrbL plays a key role in amino acid acylation and that the acyl transfer takes place before assembly of the peptide chain. Surprisingly, despite the importance of the ornibactins, the absolute configuration of the *N*-acyl chains has remained elusive. Indeed, isolation of the free fatty acid proved to be more challenging than expected. Even so, optimization of hydrolysis conditions (1 M H_2SO_4 at 80 °C),[17] followed by separation via an SPE column granted access to the pure hydroxy fatty acids. The absolute configuration of the main component, 3-hydroxyoctanoic acid (**4**) was analyzed using Mosher's method.[18] Despite overlapping methylene signals, the ^{1}H NMR data unequivocally showed that the configuration is *R* (Figure 2B).

Taken together, the different chemical structures of ornibactin and malleobactin arise not only from the presence or absence of the acyltransferase genes but also from different adenylation domain specificities. Which conclusions can be drawn for the evolutionary connection of both gene clusters? Regarding the order of adenylation domain mutation and gain or loss of the acyltransferase genes, four hypotheses can be proposed (Figure 3).

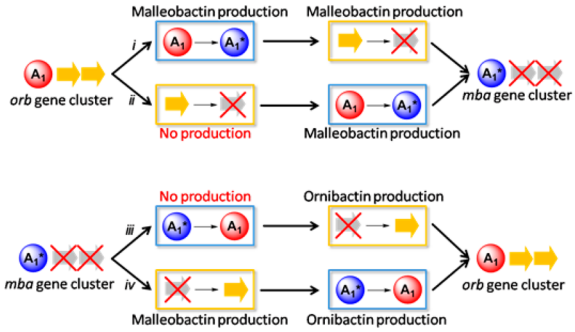

Figure 3. Possible evolutionary routes for the transition between *mba* and *orb* gene clusters and *vice versa*. Either mutation in the adenylation domain specificity (blue boxes) can occur first, followed by gene loss/gain (yellow boxes) (i and iii) or *vice versa* (ii and iv).

As there are no clear hints whether or not *orbK* and *orbL* have been acquired by horizontal gene transfer, such as a different GC content or close homologues in distant species, the evolutionary direction cannot be predicted. Therefore, *a priori* all hypotheses have to be considered as possible. However, natural products generally grant evolutionary advantages,[19] especially siderophores, which allow growth in adverse, iron-deprived environments. Therefore, all scenarios

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that lead to a temporary loss of siderophore production can be ruled out, providing that no other siderophore can make up for the loss. Although most *Burkholderia* species produce at least one additional siderophore, pyochelin,[9,20] it is generally accepted that all of these siderophores have unique roles under specific environmental conditions and cannot cross-complement each other.[21] As exchange of the adenylation domain without coexpression of acyltransferase genes abolished both malleobactin and ornibactin production (Figure 2A, trace ii), it can be assumed that routes (ii) and (iii) (Figure 3) would result in a substantial loss of fitness and can therefore be disregarded.

Both remaining hypotheses (i) and (iv), on the other hand, cannot be experimentally validated or nullified. However, we propose that route (i) is more plausible than route (iv) for several reasons. Route (iv) starts with the gain of acyltransferase genes; while this event caused the formation of the acylated amino acid (**3**), the production of malleobactin was not influenced (Figure 2A, trace vi). Although this change does not reduce the fitness of the producing organism, it neither provides any evolutionary advantage. It seems unlikely that the bacterium acquires unnecessary genes by horizontal gene transfer before the change of A domain specificity, which would render the gene products useful. One may argue that the acyltransferase genes are derived from paralogous genes from primary metabolism.[12] However, in *B. mallei* no homologous genes can be found. A plausible driving force for route (iv) can therefore not be inferred. In stark contrast, route (i) would involve a spontaneous mutation of the adenylation domain specificity that would directly lead to the formation of malleobactins. Subsequent loss of the acyltransferase genes would be beneficial by saving energy and resources, improving the supply of the correct substrate for the assembly line (Figure 2C, Scheme S1) and reducing the genome size.[22] Although most *Burkholderia* species maintain relatively large genomes due to the variety of lifestyles, these genomes are still highly optimized regarding unnecessary genetic material.[23−26] Another point that supports route (i) is the fact that hydroxamate siderophores are well-known, while malleobactin A derives from a highly unusual unprotected hydroxylamine.[9] Overall, the most parsimonious scenario would involve the ornibactin gene cluster as the evolutionary predecessor. It is well conceivable that there are similar evolutionary processes for related pathways in other species.

In summary, we modified a malleobactin-producing strain from the *B. mallei* family to produce the closely related siderophore ornibactin from the human pathogenic *B. cepacia* family. To our knowledge, this is the first example for the targeted manipulation of an NRPS gene cluster to produce another known natural product. Additionally, we solved the last stereochemical question of the ornibactins, showed that amino acid acylation precedes peptide assembly, and demonstrated that OrbL alone is capable of producing all ornibactins. Our results contribute to understanding the evolution of natural product biosynthesis gene clusters and of virulence traits of human pathogenic bacteria.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details, colony PCR of mutant strains, amino acid sequence alignments of adenylation domains, isolation procedures for **3** and **4**, NMR data and spectra of **3**, Mosher analysis of **4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

The authors declare no competing financial interests.

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