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Merging Chemical Ecology with Bacterial Genome Mining for Secondary Metabolite Discovery

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Abstract

The integration of chemical ecology and bacterial genome mining can enhance the discovery of structurally diverse natural products in functional contexts. By examining bacterial secondary metabolism in the framework of its ecological niche, insights can be drawn for the upregulation of orphan biosynthetic pathways and the enhancement of enzyme substrate supply to illuminate new secondary metabolic pathways that would otherwise be silent or undetected under typical laboratory cultivation conditions. Access to these new natural products (*i.e.*, the chemotypes) facilitates experimental genotype-to-phenotype linkages. Here, we describe select functional natural products produced by *Xenorhabdus* and *Photorhabdus* bacteria, with experimentally linked biosynthetic gene clusters, as illustrative examples of synergy between chemical ecology and bacterial genome mining in connecting genotypes to phenotypes through chemotype characterization. These Gammaproteobacteria share a mutualistic relationship with nematodes and a pathogenic relationship with insects, and in select cases, humans. The natural products encoded by these bacteria distinguish their interactions with animal hosts and other microorganisms in their multipartite symbiotic lifestyles. Though both genera have similar lifestyles, their genetic, chemical, and physiological attributes are distinct. Both undergo phenotypic variation and produce a profuse number of bioactive secondary metabolites. We provide further detail in the context of regulation, production, processing, and function of these genetically encoded small molecules with respect to their roles in mutualism and pathogenicity. These collective insights more widely promote the discovery of *atypical* orphan biosynthetic pathways encoding *novel* small molecules in symbiotic systems, which could open new avenues for investigating and exploiting microbial chemical signaling in host-bacteria interactions.

Keywords

chemical signaling; natural products; secondary metabolism; biosynthesis; structure elucidation; symbiosis

Introduction

Chemical ecology is an interdisciplinary field that seeks to identify and elucidate the functions of natural molecules in mediating biotic and abiotic interactions among organisms and their surrounding environments. Hartmann has detailed the field's lost origins, describing early pioneering work in the mid-19th century primarily by Ernst Stahl, whose

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This manuscript is dedicated to Sir David Hopwood on the occasion of his 80th birthday.

field observations of plant-herbivore interactions led him to study the chemical defenses of plants in the laboratory [55]. But the concept of chemical ecology remained largely ignored until the 1950s mainly due to the primitive belief that the majority of secondary metabolites were waste products from primary metabolism. The landmark paper by the entomologist Gottfried Fraenkel (1949) played a major role in propelling chemical ecology to the forefront, highlighting plant-insect studies and the evolutionary adaptation of plant secondary metabolites to either deter or attract insects [39]. This ever-expanding field has come to encompass all dynamic small molecule functional interactions among organisms, including vertebrates [92, 110], invertebrates [5, 7, 23, 54, 58, 103, 117], and microorganisms [91, 106, 116, 121]. These genetically encoded small molecules often represent *extracellular* extensions of genomic instructions and the intercellular transfer of *information*, which are regulated accordingly and are frequently "silent" in common laboratory media. As such, chemical ecology does not need to be mutually exclusive from microbial genome mining and synthetic biology approaches for secondary metabolite stimulation and discovery. Rather, the combination of these disciplines places natural products in genomic, regulatory, functional, and ecological contexts, dramatically enhancing the productivity and efficiency of emerging genes to molecule discovery platforms.

Over the last decade, the continuous flood of microbial genome sequencing has revealed a much greater biosynthetic potential for the synthesis of diverse and novel secondary metabolites than previously appreciated [11, 51, 67, 95, 115, 135, 138, 141]. The majority of secondary metabolic pathways observed in genomic analysis are "orphan" and encode "cryptic" metabolites. In this context, cryptic simply means that an encoded molecule has not yet been correlated to its biosynthetic pathway. While unknown secondary metabolite pathways are occasionally referred to as being "silent," this term implies some regulatory knowledge of the pathway and its usage should only be applied when supporting experimental regulatory data is available. Indeed, many expressed pathways producing molecules ranging from inert to highly unstable are simply undetected due to incompatible extraction, processing, and/or analytical techniques. Functional approaches, such as bioassay-guided fractionation, remain robust methods to empirically optimize the production, extraction, isolation, and structural characterization of *unknown* natural products with often-unpredictable chemical and biological properties. Here in lies a key advantage to focusing on symbiotic systems for natural product discovery, as experimental conditions and screening parameters can be leveraged within the physiological and ecological contexts of a given symbiosis.

To establish synergy among chemical ecology, natural product discovery, and genome mining, fundamental evolutionary mechanisms in microbial functional adaptation should be considered. Microbial genomes are dynamic and major phenotypic differences among related species are often associated with the evolutionarily malleable secondary metabolic pathways. In bacteria and fungi, the genes responsible for the biosynthesis, regulation, resistance, and transport of a given metabolite are frequently encoded on a contiguous stretch of the genome (*i.e.*, "gene cluster"), facilitating experimental genotype-to-phenotype correlations and the discovery of functional gene cluster-specific small molecules (also known as a chemotype). These secondary metabolic gene clusters, which are often not essential for cellular growth in ideal growth conditions, represent highly adaptable genetic elements that evolve through genetic mutation, gene duplication, gene deletion, sequence migration, and genome rearrangements through periods of genetic drift and natural selection [15, 38, 63, 88, 137].

While gene duplication and functional divergence substantially contribute to natural product structural diversification within a given species (*e.g.*, the genetic expansion of polyketide synthases in fungal species) [72], the exchange of genetic information between organisms

can be a central player in mediating host-bacteria interactions. For example, the acquisition of a new encoded trait via the horizontal gene transfer of chromosomal, plasmid, or phage sequences can rapidly redefine the landscape available to a microorganism [98]. The resulting genomic islands can contribute to functional adaptation by enhancing pathogenicity, symbiosis (mutualistic or parasitic traits), fitness, or drug resistance [32, 53]. Functional attributes that can migrate on genomic islands are wide ranging and include virulence factors, adhesins, invasins, modulins, effectors, secretion systems, iron acquisition systems, protein toxins, and in particular, bioactive small molecules. An important aspect of chemical ecology focuses on the microbial acquisition of orphan biosynthetic pathways involved in overcoming new challenges (*e.g.*, functional small molecule contributions to host-bacteria interactions). These secondary metabolic pathways vary widely across species and even individual strains. In this regard, natural products themselves can promote bacterial niche differentiation, and ultimately, contribute to speciation [64, 73, 104].

To highlight our points, natural products from the two well-studied Gammaproteobacteria genera, *Xenorhabdus* and *Photorhabdus*, will be reviewed in greater detail. These bacteria produce a diverse array of small molecules that play an assortment of biological roles in regulating their multipartite symbioses [5, 7], in which the bacteria serve as both mutualists of specific nematode hosts and pathogens of a variety of insect hosts [17, 36, 48, 49, 59, 93, 112, 130]. Interestingly, *Photorhabdus asymbiotica* also made the evolutionary leap from invertebrate to human pathogenesis. In addition to infecting insects, *P. asymbiotica* represents an emerging human pathogen that causes soft tissue and blood infections [20, 45, 47, 124, 130, 134, 136]. Descriptions of the genomes from several *Xenorhabdus* and *Photorhabdus* species have been published [14, 34, 44, 56, 75, 99, 136], revealing an enormous potential for secondary metabolite synthesis. In part, the natural products encoded by these bacteria distinguish their interactions with animals and other microbes during their variable life histories. Here, we describe select natural products, with experimentally linked gene clusters, and highlight their likely functional aspects in the *Xenorhabdus* and *Photorhabdus* lifecycles. The examples are used to illustrate the synergy between chemical ecology and bacterial genome mining approaches for small molecule discovery in functional contexts, which in practice, could be more widely applied across diverse symbiotic systems. We then conclude by positing that such collective insights could facilitate the discovery of *atypical* orphan biosynthetic pathways encoding *novel* functional small molecule structural classes and/or features, which could open new avenues for investigating and exploiting microbial chemical signaling in host-bacteria interactions.

Xenorhabdus and *Photorhabdus* multipartite lifestyles

Xenorhabdus species are prolific antibiotic-producing Gammaproteobacteria that share a mutualistic relationship with nematodes in the *Steinernema* genus, whereas the related bioluminescent *Photorhabdus* species share a relationship with *Heterorhabditis* nematodes [17, 36, 48, 49, 59, 93, 112, 130]. The bacterium-nematode complexes are highly efficient at infecting and killing a wide range of insect larvae in the soil, leading to their commercialization as biological control agents of agricultural pests. In addition to producing immunomodulators to circumvent the insect's innate immune system and insecticides that assist in the insect's demise, the bacteria encode an armament of bioactive molecules and antibiotics to modulate their own development utilizing the insect's biomass as an energy source, influence the development of the nematode, and protect their decomposing insect prey from competing bacteria and fungi in the combative soil microenvironment. For *P. asymbiotica*, additional molecules are likely produced to circumvent the human immune system and establish niches in the soft tissue and bloodstream [20, 45, 47, 124, 130, 134, 136].

Despite their overall similarities, physiological, genetic, and genomic comparisons across genera indicate that *Xenorhabdus* and *Photorhabdus* underwent divergent evolution leading to convergent lifestyles [14, 34, 44, 49, 56, 75, 99, 136]. Consequently, the bacteria collectively produce a treasure trove of molecules for at least five biomedically-relevant objectives – immunomodulators, insecticides, developmental modulators, antibacterials, and antifungals – and are strikingly comparable to *Streptomyces* in regards to their secondary metabolic repertoire. Currently, there are 22 recognized species of *Xenorhabdus* and three species of *Photorhabdus* [93]. Combined with the intraspecies and interspecies chemical variability observed to date, they continue to represent a rich source of small molecules with potential commercial value. Unlike the poorly understood interactions that occur in complex microenvironments, such as in bulk soil, an understanding of the simpler animal-bacteria systems facilitates the upregulation of molecules for discovery in the lab and the functional connection(s) for which the molecules evolved to fulfill.

Xenorhabdus and *Photorhabdus* phenotypic variation and regulation

In keeping with their alternative lifestyles in multiple animal hosts, *Xenorhabdus* and *Photorhabdus* species stochastically undergo phenotypic variation, which manifests in at least two highly distinct forms, primary and secondary. These forms, which are controlled by mechanistically distinct regulatory systems between genera, can be distinguished on Petri dishes simply by eye using dye supplements [1]. The primary form is associated with the secretion of a variety of extracellular products, including hemolysins, proteases, lipases, and antibiotics. Many of these factors are needed for both mutualistic nematode development and insect pathogenicity. Understanding the mechanisms of phenotypic variation and small molecule regulation are important for bridging the *Xenorhabdus/Photorhabdus* orphan biosynthetic gene clusters to the molecules they produce in a native context and the phenotypic effects they regulate in the symbiosis.

Early investigations of *Xenorhabdus nematophila* and *Photorhabdus temperata* revealed that the two genera exhibit remarkably dissimilar downstream regulatory mechanisms for antibiotic biosynthesis [21, 66]. In *X. nematophila*, a leucine-responsive regulatory protein (Lrp) is a positive regulator of phenotypes associated with the primary form (*i.e.*, a Δ*lrp* mutant lacks antibacterial zones of inhibition) [21]. The upstream regulatory mechanisms that control phenotypic variation leading to the primary form in *Xenorhabdus* are currently unclear, although it has been suggested that epigenetics could be an important player [122]. In *P. temperata*, a LysR-type transcriptional repressor (HexA) contributes to the regulation of primary form features, such as antibiotic activity (*i.e.*, a Δ*hexA* mutant enhances antibacterial zones of inhibition) [66]. Despite the regulatory disparities – positive versus negative regulation – both *Xenorhabdus* and *Photorhabdus* primary forms produce relatively high titers of general insecticidal, antibacterial, and antifungal activities during insect infection [60, 61, 84], providing an advantage to the nematode-bacteria complex during pathogenicity.

More recent genetic, pulse-chase, and microscopy experiments have provided further important insights into the mechanism of stochastic form switching in *Photorhabdus* [119]. A mutant library search for genes required in nematode colonization led to the identification of a fimbrial locus, maternal adhesion defective (*mad*), in *Photorhabdus luminescens* [118]. The locus was controlled by an ON/OFF invertible promoter switch, the *madswitch* (**Fig. 1**) [119]. Depending on the orientation of the 257-bp promoter region, which was flanked by 36-bp inverted repeats, the bacteria toggled between a pathogenic primary form (“P-form”) and a less prevalent small colony variant secondary form (“M-form”). The P-form robustly produces antibiotics, insect virulence factors, and mutualistic nematode factors. Strikingly, the small colony variant M-form, which was avirulent towards insects, selectively adhered

to posterior intestinal cells of the maternal nematode to initiate mutualistic association ("M-form" bacteria). MadJ was identified in the locus as the transcriptional activator responsible for regulating the M-formation program. An understanding of the form switching mechanism enabled the bacteria to be engineered in either genetically-locked state [119]. Locked forms could be particularly helpful for novel bioactive small molecule discovery, as opposed to identifying molecules from minority cell variants in mixed bacterial populations. Locked forms also provide a unique opportunity to investigate the metabolic status between pathogenic and mutualistic initiation states of the infectious bacteria. Indeed, LC/MS analysis showed that the locked P-form robustly produced bioactive small molecules, such as bacterial stilbenes and anthraquinones, and microscopy confirmed that this engineered strain was incapable of generating M-form revertants during cultivation. Additionally, transcript microarray analysis revealed few upregulated orphan biosynthetic genes in the locked M-form during exponential growth relative to the P-form, suggesting that select bioactive small molecules could contribute not only in combative P-form infections, but perhaps also as currently unknown M-form colonization factors [119]. Small colony variants related to the M-form are common in chronic human infections [108], such as in cystic fibrosis, and it would be prudent to identify small molecules that might contribute to persistent bacterial colonization of animal hosts in general.

Parallel functional small molecule screening platforms

The innate immune systems of insects share similarities with mammalian innate immunity [70, 125]. Consequently, some bacterial virulence strategies utilized in invertebrate pathogenesis can be evolutionarily redeployed for vertebrates, increasing the utility of invertebrate model systems in the lab [132]. The dual insect-human pathogen *P. asymbiotica* represents an excellent example of bacterial virulence mechanisms being employed in insect and/or human hosts [20, 45, 47, 124, 130, 134, 136]. It is hypothesized that the *Heterorhabditis* nematode can actively penetrate intact and healthy human skin to deliver the pathogen [46]. Once delivered, *P. asymbiotica* effectively outmaneuvers the human immune system, leading to soft tissue and blood infections [20, 45, 47, 124, 130, 134, 136].

A parallel screening strategy, Rapid Virulence Annotation (RVA), was carried out to identify bacterial genomic islands in *P. asymbiotica* that contribute to host toxicity across diverse taxa – insects, nematodes, protozoa, and mammalian macrophage cell lines [131]. To identify small molecules *toxic* to select eukaryotes, RVA requires a heterologous bacterial host capable of expressing orphan biosynthetic pathways from genomic libraries in the presence of the eukaryote host. For *P. asymbiotica*, expression of a cosmid library in *Escherichia coli* led to the sequence identification of eight NRPS and two PKS orphan biosynthetic gene clusters that resulted in toxic phenotypes for the eukaryote hosts, suggesting that the orphan pathways could contribute to virulence. One of the orphan pathways toxic to insects and mammalian cells was predicted to synthesize antitumor glidobactin analogs based on bioinformatics [131]. Using a homologous recombination cloning strategy, a related biosynthetic pathway in *P. luminescens* was overexpressed in *E. coli* and experimentally shown to produce the glidobactin analogs, luminmycins A-C (**Fig. 2**, Structures **1, 3-4**) [3, 41], where luminmycin A exhibited cytotoxicity against a colon cancer cell line in the nanomolar IC_{50}-range [3]. Osmotic culture shock combined with a NMR-based proteasome inhibitory assay, led to the identification of glidobactin A (**5**) and the highly potent antitumor cepafungin 1 (**6**) in wildtype *P. luminescens* cultures [120]. Production of glidobactin A (**5**) and luminmycins A and D (**1-2**) were confirmed in *P. asymbiotica* cultures using a defined medium previously shown to induce secondary metabolism in some *Streptomyces* species [123]. These small molecule proteasome inhibitors were also expressed in bacterium-infected crickets, keeping with one of their regulatory niches and functional attributes in virulence. In principle, the manipulation of

sequence inputs, engineered heterologous expression strains, and alternative functional screening outputs could readily be tailored to dramatically facilitate genotype to chemotype to phenotype correlations in any related parallel natural product discovery platform.

Functional small molecules from primary-form bacteria

Earlier structural investigations of bioactive metabolites produced by *Photorhabdus* primary form cultures led to the discovery of anthraquinone polyketides (*e.g.*, **Fig. 2**, Structures **7-8**) and bacterial stilbenes (*e.g.*, **Fig. 3**, Structure **34**) [113]. The anthraquinone pigments, which are thought to provide anti-omnivory properties for the bacteria-nematode-insect complex [19], were linked to their discretely expressed (type II) polyketide synthase gene cluster by gene deletion, isotopic labeling, and product analysis [8]. The major antibiotic molecules present in laboratory cultures were identified as bacterial stilbenes [77, 113], which are disseminated throughout the *Photorhabdus* genera. The stilbenes are multipotent polyketide-phenylpropanoids, exhibiting antimicrobial activity against Gram-positive bacteria and fungi [61], harboring moderate inhibitory activity against the metalloenzyme phenoloxidase, an important terminal component of the insect's innate immune system [35], and crucially contributing to host nematode development [65]. A fragmented biosynthetic pathway (*i.e.*, not on a contiguous stretch of the genome) was identified via genetics and product analysis. It was proposed that two β-keto-acyl-derivatives (**32** and **33**) were condensed in a head-to-head manner, leading to the stilbene scaffold (**Fig. 3**) [65]. Intriguingly, this biosynthetic strategy is much different than the type III PKS strategy common to plant stilbene biosynthesis, indicating convergent evolution to similar structural outcomes.

Identification of regulators governing symbiotic interactions can also promote the discovery of small molecules involved in specific lifestyle transitions. Microbiologists have identified a panoply of regulatory elements in *Xenorhabdus/Photorhabdus*, and many other bacterial symbionts, capable of producing bioactive small molecules with pharmacological value. There are far fewer examples, however, in which natural product chemists examined the chemical potential of regulatory mutants directly connected to bacterial lifestyle decisions. In *P. luminescens*, for example, insertional inactivation of *hexA*, the repressor previously identified to be involved in regulating primary form characteristics [66], led to an upregulation of previously described and new bacterial stilbenes [71]. A newly identified dihydrostilbene derivative (**31**) was shown to undergo oxidation in aerobic conditions to form the major aromatic stilbene (**34**) (**Fig. 3**). Such easily oxidized metabolites could assist in overcoming oxidative stress from the insect's innate immune system [71]. L-2,5-Dihydrophenylalanine (**30**), an antimetabolite of phenylalanine biosynthesis, was identified in *P. luminescens* cultures and shown to be the substrate for dihydrostilbene in isotopic feeding studies [25]. A recently characterized nonaromatizing prephenate decarboxylase common to secondary metabolism [80, 81] was identified in a separate orphan biosynthetic pathway. Genetic, small molecule, and biochemical analyses determined that this previously orphan pathway initiated the biosynthetic process from prephenate to L-2,5-dihydrophenylalanine (**Fig. 3**) [25]. With over 500 other nonproteinogenic amino acid derivatives known, the continued elucidation of their underlying gene clusters will aid not only in the *in vivo* engineering of new nonribosomal peptides, but also increasingly in the ribosomal protein engineering field [127].

Related to the bacterial stilbene biosynthesis, two fatty acid precursors (**Fig. 3**, Structures **35-36**) are condensed by a ketosynthase homolog to generate 2,5-dialkylcyclohexane-1,3-diones (CHDs, *e.g.*, enzyme-tethered intermediate **37**) [42]. Separate enzymes, including an aromatase, were required to oxidize the CHD scaffold to its corresponding resorcinol scaffold (**38**). The CHD natural product class was recently introduced, when they were discovered to serve as orchid sex pheromones [40]. Phylogenetic analysis indicated that the

biosynthesis of CHDs is widespread and enriched in bacteria that interact with eukaryotes [42], supporting unresolved regulatory functions in various eukaryote-bacteria associations.

It has long been known that the alteration of growth conditions in the lab profoundly affects the production of secondary metabolites (*e.g.*, as applied in the one strain-many compounds (OSMAC) approach [6]). Symbiotic systems provide immediate insights to guide cultivation parameters for secondary metabolite stimulation and discovery. Addition of hemolymph (insect blood) to *Xenorhabdus* and *Photorhabdus* laboratory cultures substantially stimulated the production of the previously noted stilbenes and anthraquinones in primary form cultures of *P. luminescens* TT01 and of a variety of new and previously described indole-containing antibiotics in *X. nematophila* [24]. Bioassay-guided fractionation of the hemolymph for the identification of antibiotic-induction factors identified L-proline and osmotic stress as principal induction factors. Addition of L-proline at low millimolar (mM) concentrations stimulated antibiotic production, while deletion of metabolic or osmotic proline transporters in *P. luminescens* dramatically reduced stilbene virulence factor production. Analysis of various insect hemolymph samples confirmed the presence of free amino acid L-proline between 3-73 mM, demonstrating a physiologically-relevant response [24]. While individual bacterial species and strains behave significantly different in regards to bioactive small molecule production, we regularly observe higher antibiotic titers in primary form *Xenorhabdus/Photorhabdus* cultures supplemented with free amino acids over typical peptide supplements (*e.g.*, tryptone).

L-Proline media supplementation enabled the upregulation and discovery of other bioactive metabolites and their assignment to previously orphaned pathways. Particularly in *X. nematophila*, it led to the identification of a diversified family of NRPS cyclodepsipeptides xenematides A-D (**9-12**) [26] and a new isocyanide- and amidoglycosyl-functionalized natural product, rhabduscin (**14**) [24]. The antibacterial xenematide A (**Fig. 2, 9**) had been previously characterized from primary form *X. nematophila* cultures [74], and its absolute configuration was determined by total synthesis [62]. Both *Xenorhabdus* and *Photorhabdus* species produce a variety of other nonribosomal peptides with a variety of biological activities. For example, a family of lysine-/arginine- rich cyclic peptides, the PAX peptides (**Fig. 2, 15-27**), were identified from *X. nematophila*, which harbor potent antifungal activity [43, 52].

The less prevalent isocyanide natural products have recently been identified from a number of cultured and environmental bacteria [10]. L-proline stimulated rhabduscin (**14**) production by over an order of magnitude in laboratory cultures of *X. nematophila*, facilitating its structural determination [24]. Rhabduscin exhibited potent nanomolar-level inhibition against the insect's innate immune enzyme, phenoloxidase, and its biosynthetic pathway was critical for infection [27]. At high bacterial inoculum, rhabduscin deletion mutants were markedly impaired in infectivity, and at lower inoculum, the mutants were avirulent. In contrast, wildtype bacterium killed all of the larvae at all cell inoculum concentrations, indicating that the small molecule pathway could represent a make-or-break point for pathogenicity. While rhabduscin was identified, in part, as a diffusible product in the spent culture medium, the molecule was also found localized to the outer bacterial lipopolysaccharide by stimulated Raman scattering microscopy. The inhibitor's location at the cell's periphery provides a spatially appropriate and high local concentration to defend the bacteria from the insect's phenoloxidase response. The rhabduscin gene cluster has been identified in genomes from both *Xenorhabdus* and *Photorhabdus* species, including the human pathogen *P. asymbiotica*. The isocyanide-aglycone intermediate (**13**) was also identified in *Vibrio cholerae* pathogens [27]. *V. cholerae* is thought to exploit arthropods as reservoirs to persist in endemic areas as well as vehicles to spread to alternative locales [109]. Consequently, isocyanides could potentially contribute in part to cholera spread

through its potent inhibition of phenoloxidases in arthropod hosts and/or inhibition of other metalloenzymes in the human innate immune system [27].

Gene duplication of primary metabolic genes and functional divergence to secondary metabolic functions was likely an early evolutionary mechanism leading to the expansion of an enzyme class that utilizes α-keto acid substrates in secondary metabolism. The pyruvate dehydrogenase complex (E1, E2, and E3) in primary metabolism is an important connector of glycolysis with the TCA cycle, converting the simple α-keto acid pyruvate to acetyl-CoA [105]. Other α-keto acids, such as those processed by the related branched chain α-keto acid dehydrogenase complex (BCKAD) [100], similarly yield their related CoA-products. Secondary metabolic genes have been frequently observed in genome databases clustered with or fused to related pyruvate dehydrogenase-like subunits. In *X. nematophila*, rather than release the free acyl-CoA, the acyl-group is likely transferred directly to the CoA-derived phosphopantetheine arm of a PKS acyl-carrier protein to initiate synthesis of branched-chain fatty acids and the streptogramin-type antibiotic pristinamycin IIa (**Fig. 2**, Structure **28** with starter unit bolded) [9]. In this example, the *Xenorhabdus* gene cluster is flanked by transposase sequences supporting its acquisition from an unidentified extrachromosomal source and its repurposing for the *Xenorhabdus* lifestyle. Similar chemistry had also been observed from metagenomic DNA sources in the construction of branched-chain fatty acid substrates in select N-acyl-amino acid biosynthesis [22]. Pristinamycin IIa, a well-known *Streptomyces* antibiotic, is a precursor for the semi-synthesis of one active constituent of the two-component commercial antibiotic synercid [18], and the pristinamycin gene cluster in *Streptomyces pristinaespiralis* was recently reported [83].

Proteolytic Processing of Nonribosomal Peptides

An *X. nematophila* compound thought to play a major role in insect cadaver sterility is the water-soluble benzopyran-1-one isocoumarin derivative xenocoumacin 1 (**Fig. 4**, Xcn 1, **39**). Both Xcn 1 and xenocoumacin 2 (Xcn 2, **40**) were first isolated from insect cadavers and as major compounds produced from the culture broth of *Xenorhabdus* stains [84]. Xcn 1 exhibits strong antibacterial activity against Gram-positive [140] and Gram-negative bacteria, antifungal activity, and potent antiulcer activity [85]. On the other hand, Xcn 2 shows substantially reduced bioactivity. Derivates of Xcn 1 and 2 have also been identified at trace amounts, though their bioactivity has not been reported [111].

The 34-kb biosynthetic gene cluster associated with the production of xenocoumacins (Xcn) contains 14 genes (*xcnA*-*xcnN*), which includes two NRPS (*xcnAK*), three PKS (*xcnFHL*), and nine accessory genes [33, 111]. The PKS and NRPS genes *xcnAKFHL* are necessary for Xcn 1 synthesis, and the accessory genes *xcnBCDE* are specifically involved in the biosynthesis of the extender unit hydroxymalonyl-ACP [13, 33, 111]. The conversion of Xcn 1 into Xcn 2 is due to *xcnM* and *xcnN* genes, which encode proteins homologous to saccharopine dehydrogenases and fatty acid desaturases, respectively [111]. When Xcn 2 production is attenuated, an increase in Xcn1 is observed along with a 20-fold reduction in cell viability, suggesting that conversion of Xcn 1 to Xcn 2 is a resistance mechanism utilized by the bacteria to avoid self-toxicity [33].

A prodrug activation mechanism was recently identified in the biosynthesis of Xcn 1 from *X. nematophila* [30]. An in-frame deletion of peptidase gene *xcnG* produced five xenocoumacin-related compounds. The five identified pre-xenocoumacins (**Fig. 4**, PXCN, **41**-**45**) contained the Xcn 1 chemical core structure bound to a unit at the N-terminus comprising of a D-asparagine amino acid attached to one of five different acyl chains. Interestingly, these PXCN compounds did not exhibit antimicrobial activity. The *xcnG* gene encodes a protein with an N-terminal periplasmic peptidase domain that contains a signal peptide sequence and a C-terminal transmembrane domain. In this prodrug activation model

(**Fig. 4**), *X. nematophila* produces the inactive PXCN in the cytoplasm, which is then translocated to the periplasmic membrane. Once there, PXCN is cleaved into the potent antimicrobial Xcn 1 and is thought to be pumped out of the outer membrane by XcnG-ABC transporter-TolC protein complex. When present in the insect gut, Xcn 1 inhibits growth of other bacteria, allowing for a competitive advantage for *X. nematophila.* To reduce self-toxicity, Xcn 1 is cleaved by *xcnM* and *xcnN* into the less potent antibiotic Xcn 2 through the formation of the pyrrolidine ring [30].

XcnG homolog proteins have been identified in *Bacillus pumilus*, *Bacillus thuringiensis*, and *E. coli* [30], which produce amicoumacin [107], zwittermicin [79], and colibactin [97], respectively. The zwittermicin protein ZmaM is larger than the XcnG protein, containing six additional transmembrane helices and a C-terminal ABC-transporter nucleotide-binding domain. In a similar manner, ZmaM transports the pre-zwittermicin compound to the periplasm, where pre-zwittermicin is cleaved at the N-terminus into zwittermicin before being transported out of the cell [69]. Colibactin is a small genotoxin of unknown structure, biosynthesized by an NRPS-PKS gene cluster present in *E. coli* [97]. Recent studies have suggested a similar prodrug strategy in the production of colibactin. Biochemical analysis [12] and structural characterization [2] have identified an N-myristoyl-D-Asn product from the colibactin gene cluster. It is postulated that the ClbP peptidase protein cleaves a yet uncharacterized pre-colibactin molecule into the active cytotoxin(s), releasing N-myristoyl-D-Asn. Proteolytic cleavage of nonribosomal peptides is emerging as a more prominent small molecule processing mechanism in a variety of bacterial genera, implying an evolutionary preservation and likely importance in their symbiotic associations.

Secondary Metabolites and Iron Acquisition

The functional aspects of siderophores are clear for their roles in iron metabolism [89, 94, 128]. Most organisms require iron for growth and have evolved multiple mechanisms for its acquisition. In the majority of microbial habitats, bioavailable Fe(II) is oxidized to Fe(III), which leads to ferric oxide hydrate complexes ($Fe_2O_3 \times nH_2O$) and a resulting free Fe(III) concentration in the range of 10^{-9} to 10^{-18} M [89]. Siderophores, iron-chelating small molecules produced by NRPS and NRPS-independent pathways, scavenge Fe(III) to support cellular growth and serve as virulence factors in iron-limiting pathogen-host associations [28, 89, 102]. More recently, siderophores were identified as growth promoting factors that enabled previously uncultured bacteria from sediment communities to grow in the lab [29]. Such expanded cultivation of previously uncultured bacteria could significantly increase the available sources of new bioactive natural products, particularly when an understanding of the regulatory interactions between the natural products and their producers is desired [29, 76]. For microorganisms that encode siderophore-related biosynthetic pathways, biosynthesis is most often upregulated under iron-limiting conditions, and the resulting small molecule siderophores are actively secreted to scavenge Fe(III) from the extracellular environment. Consequently, growth in iron-limiting conditions in the lab has facilitated the discovery of many siderophores in a wide variety of structural classes [94]. In contrast and in keeping with the often idiosyncratic nature of secondary metabolite regulation, media supplementation with metals, such as bioavailable Fe(II), can enhance antibiotic production particularly for pathways containing common Fe(II)/α-ketoglutarate-dependent oxygenases [78, 82, 114, 129].

Siderophore chemistry plays a role in iron acquisition and antibiosis in the *Photorhabdus* lifecycle. In *P. luminescens*, a catecholate siderophore photobactin (**Fig. 2**, Structure **29**) contributes to iron acquisition and enables the bacterium to grow under iron limiting conditions *in vitro* [16]. Photobactin is biosynthetically and structurally related to known catecholate siderophores, such as vibriobactin [50] from the human pathogen *V. cholerae*

and agrobactin [101] from the plant pathogen *Agrobacterium tumefaciens*. The photobactin gene cluster contains a transposase-like element, and like many of the other secondary metabolic pathways in *Photorhabdus*, hints at its nomadic origin and its bacterial benefit in the multipartite symbiosis [16]. Unlike *V. cholerae*, in which a mutant defective in vibriobactin synthesis exhibited attenuated virulence in a mouse model [57, 139], *P. luminescens* mutants defective in photobactin synthesis did not lead to a detectable defect in supporting nematode mutualism or insect pathogenicity [16]. Rather, the TonB complex and YfeB transporter were required for metal transport in the insect and nematode environments [133]. Photobactin harbors antimicrobial activity and likely contributes to iron acquisition and antibiosis during the bacterium's competition with saprophytic bacteria near the decaying insect carcass [16]. Siderophore-related chemistry can promote antimicrobial activities against microbes lacking their cognate small molecule receptors, or in other cases, siderophore-antibiotic conjugates (sideromycins) can exploit siderophore uptake for antibiotic delivery. For *Xenorhabdus*, genome sequence analysis and secreted iron-binding activity under iron-limiting growth conditions suggest that both *X. nematophila* and *X. bovienii* are also capable of producing siderophores [14], and their roles remain unclear in the context of the symbiosis.

An approach for the discovery of novel secondary metabolism

Past and current bioinformatics strategies used to mine genomic information for natural product discovery largely focus on homology-based searches using previously described enzymes involved in antibiotic synthesis as inputs [37]. That is, you get what you search for and potentially miss biosynthetic systems that are currently unknown. For example, programs like antiSMASH utilize a collection of known antibiotic biosynthetic proteins as search criteria, which dramatically enhances initial secondary metabolite evaluations of genomic content [4, 86]. In cases of reasonably high sequence similarity, approximations of core structures can be predicted. However, many microbial genes in genome databases are annotated as encoding hypothetical proteins, and their functions cannot be predicted by sequence homology. Nor do they necessarily cluster with enzymes of perceived relevance to secondary metabolism. For some hypotheticals, functional insights can be loosely assessed through structural topology predictions that then guide subsequent *experimental* evaluations [68]. Intriguingly, many of these hypotheticals fall within genomic islands, suggesting that they might contribute to microbial functional adaptations. Some of these proteins will undoubtedly represent new biosynthetic catalysts that synthesize structurally diverse and currently unknown functional small molecules.

In addition to the homology-based searches, genome synteny (colocalization of genetic loci) among related organisms can provide a complementary view of gene content and genomic islands. Bioinformatics platforms, such as MicroScope, provide an excellent online source for visualizing genome synteny in annotated bacterial genomes [126]. Biosynthetic gene clusters, which are frequently transferred between organisms via horizontal gene transfer, often appear as acquired or lost sequence regions relative to closely related organisms in the comparative genome meta-analysis. Because many of the classical-type biosynthetic systems are “non-syntenic” across species, based on core evolutionary principles, we propose that atypical and currently unrecognized biosynthetic pathways should behave similarly. In this context, atypical biosynthetic pathways are multigenic clusters lacking genes similar to well-known biosynthetic systems and often containing multiple hypothetical enzymes. These gene clusters may encode proteins related to functionally recognizable enzymes, but they would not be exclusive to secondary metabolism. That is, these protein systems would be missed in the traditional homology-based searches for “antibiotic-like” biosynthetic proteins. As highlighted above, gene duplication of primary metabolic enzymes and functional divergence to secondary metabolic pathways is one evolutionary mechanism

for bioactive small molecule synthesis. Putative atypical biosynthetic pathways can be observed in *Xenorhabdus* and *Photorhabdus* genomes, as well as other antibiotic-producing bacteria. We expect that this mixed chemical ecology-microbial genomics approach could provide a forward avenue for discovering and characterizing these potentially new biosynthetic enzyme systems, their resulting novel small molecule products and structural features, and the new biological functions for which they were evolutionarily selected to fulfill.

Conclusion

Microbial natural products have played and continue to play a major role in the drug development process [96], as their privileged scaffolds have been crafted by functional evolutionary selection. The reader is directed to an accompanying review by Demain in this Special Issue on Genome Mining dedicated to Sir David Hopwood for an overview of pharmacologically-relevant natural products [31]. The development of high-throughput and low cost microbial genome sequencing combined with the investigative exploitation of complex host-microbe interactions and the design of newly emerging synthetic biology platforms (*e.g.*, [87, 90]) have ushered in a new era of natural products discovery that require fresh perspectives and interdisciplinary research programs. A revitalization of natural product discovery platforms in the post-genomic era shows great promise for the discovery of new functional small molecule classes and/or structural features. Chemical ecology can dramatically enhance these discovery platforms by placing orphan biosynthetic pathways and the cryptic molecules they produce in regulatory, genomic, functional, and ecological contexts.

We highlighted some illustrative natural product examples in the *Xenorhabdus* and *Photorhabdus* lifecycles, in which a greater understanding of the regulatory roles in their symbiotic lifestyle enhanced the discovery of new bioactive molecules in the lab. Additionally, their ecological contexts facilitated functional assignments that these molecules likely evolved to fulfill. With the numerous secondary metabolic genes emerging in the ever-expanding microbial genome databases, notwithstanding the increasing number of hypothetical genes providing sparse functional insights, interdisciplinary platforms to decode orphan secondary metabolic pathways and genomic islands will certainly reveal new biosynthetic catalysts involved in natural product synthesis and diversification, new evolutionarily privileged and functional small molecule structural features, and new chemical signaling events with potentially quite profound biomedical and commercial implications.

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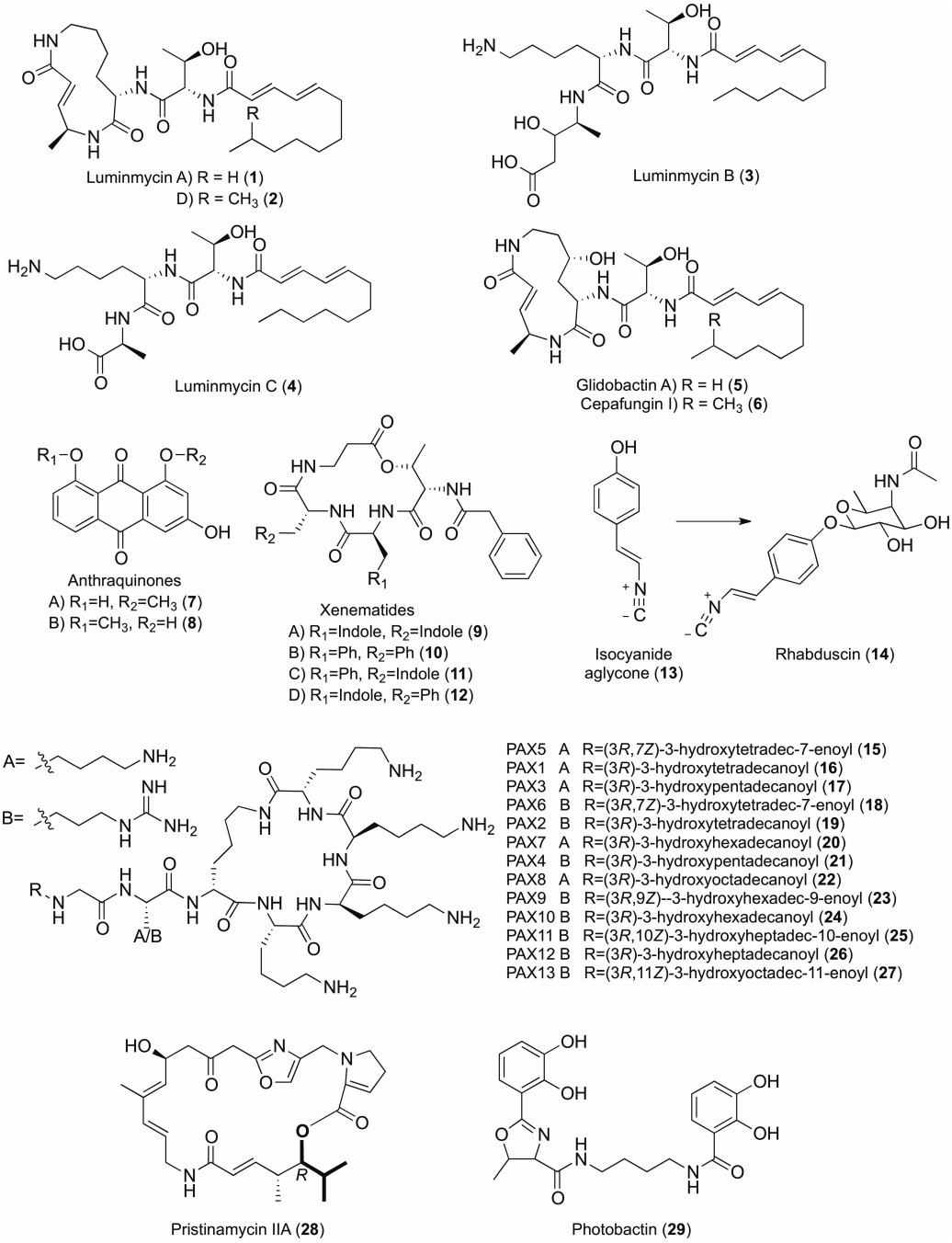

Figure 1.

Phenotypic variation in *Photorhabdus*. Promoter inversion toggles the bacterium between a pathogenic P-form and an M-form that initiates nematode mutualism. A new understanding of the genetic form switching mechanism enables engineered locked states to examine the metabolic status associated with phenotypic variation. Adapted from Somvanshi et al [62].

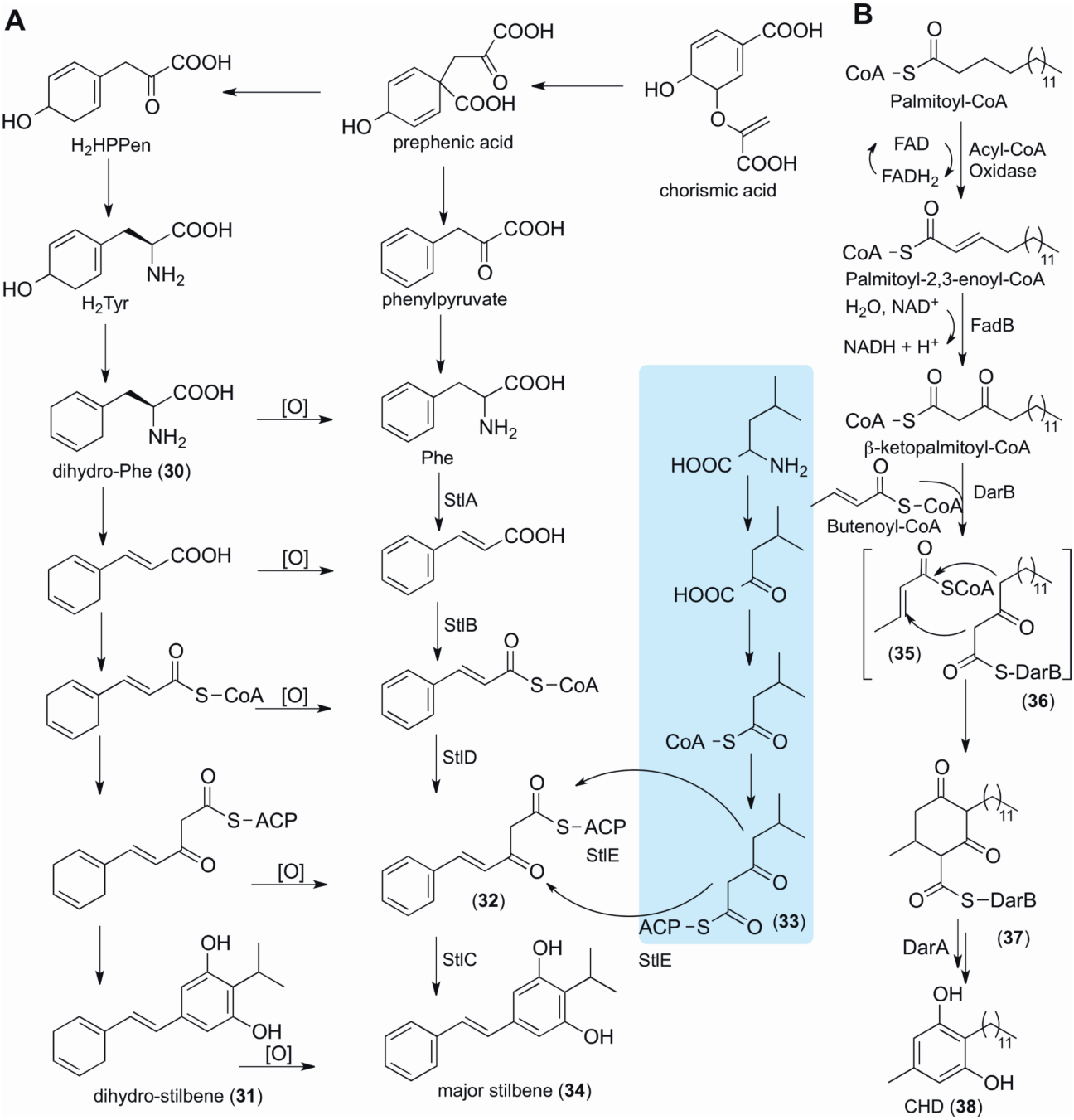

Figure 2.
Selected natural products from *Xenorhabdus* and *Photorhabdus*.

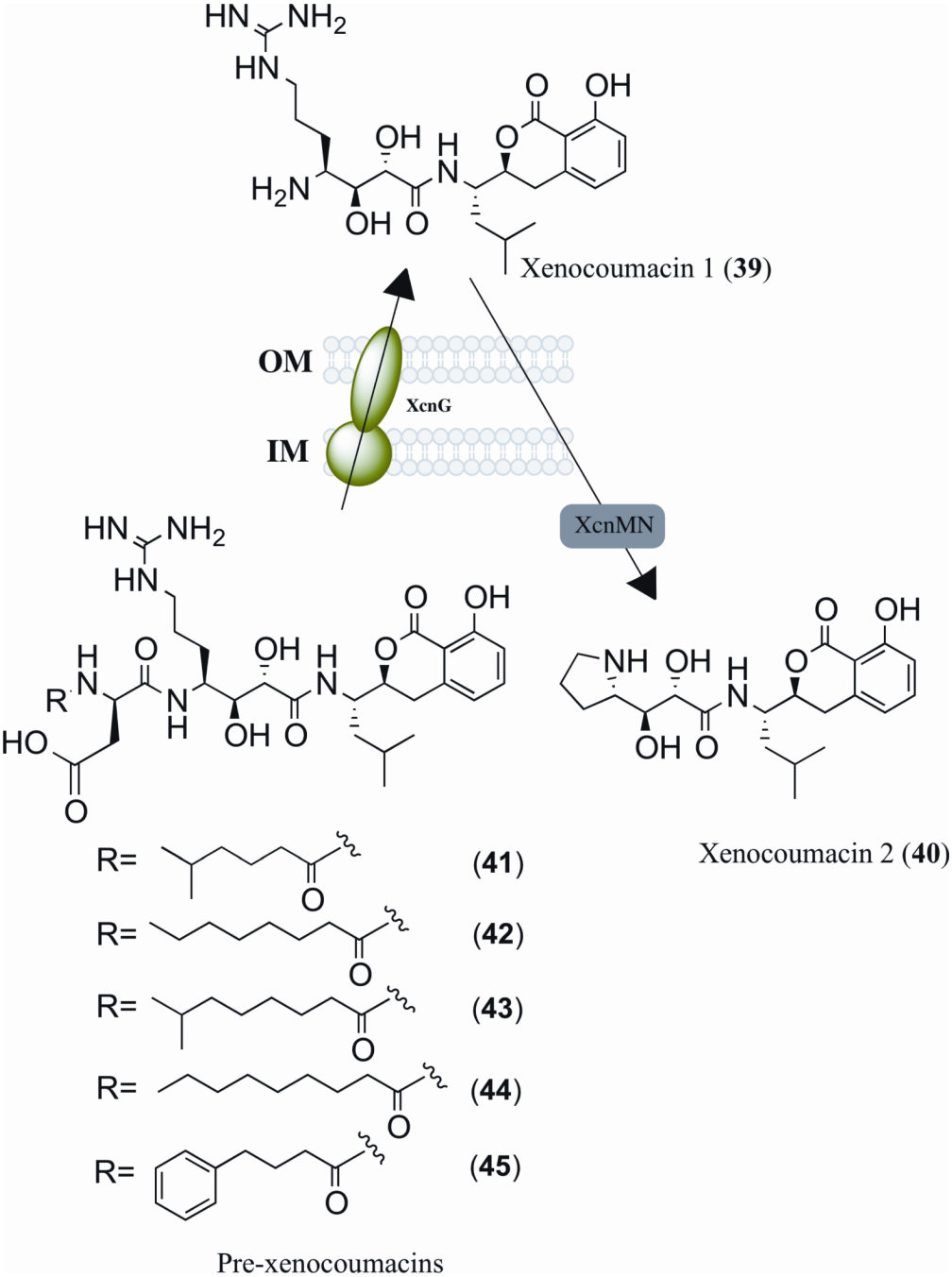

Figure 3.
Proposed stilbene and cyclohexanedione biosynthesis. Based on enzyme homology, it is plausible that stilbene biosynthesis could rather proceed as shown for cyclohexanedione biosynthesis.

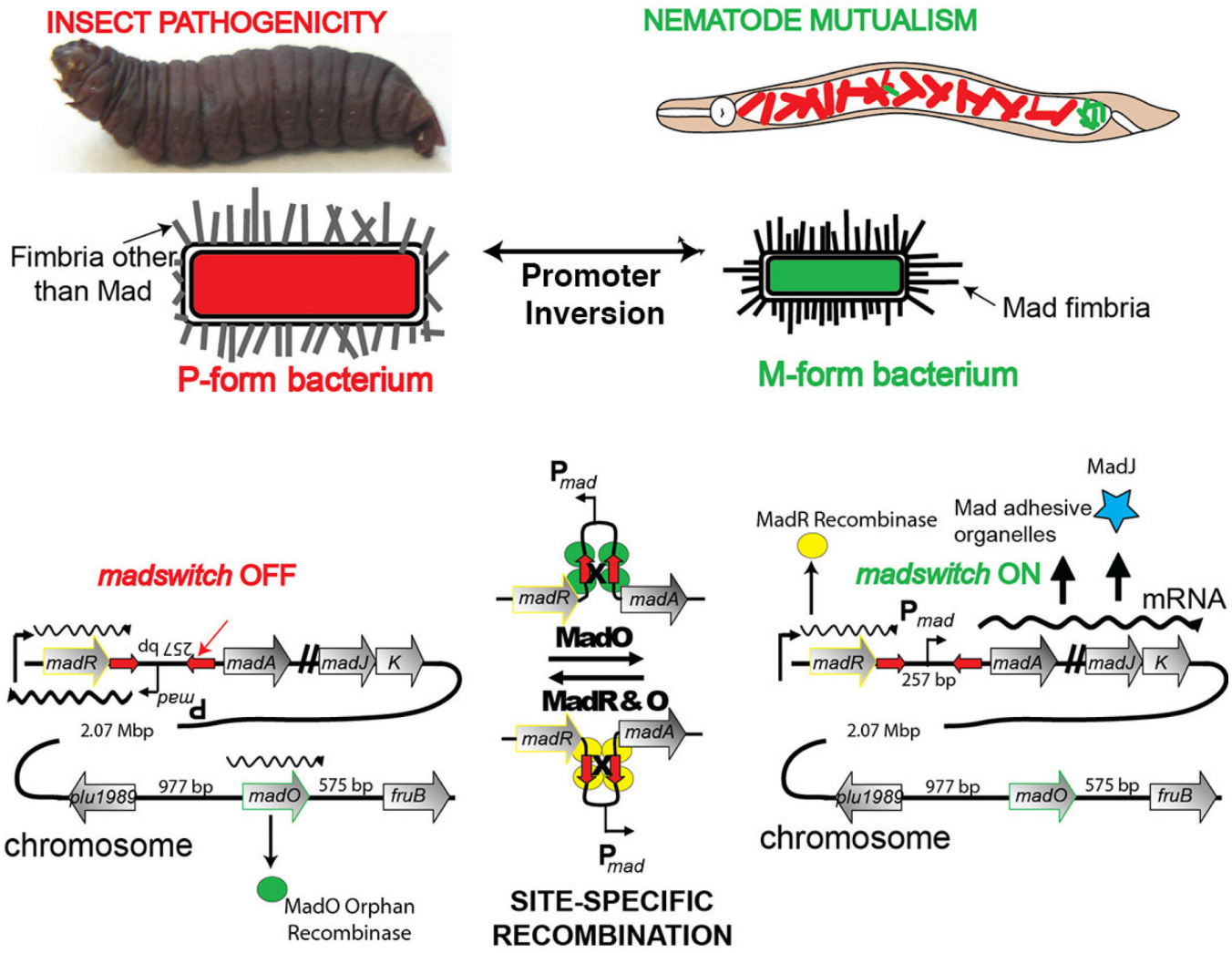

Figure 4.
Proteolytic processing in xenocoumacin biosynthesis and activation. OM, outer membrane; IM, inner membrane.