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Signaling Natural Products from Human Pathogenic Bacteria

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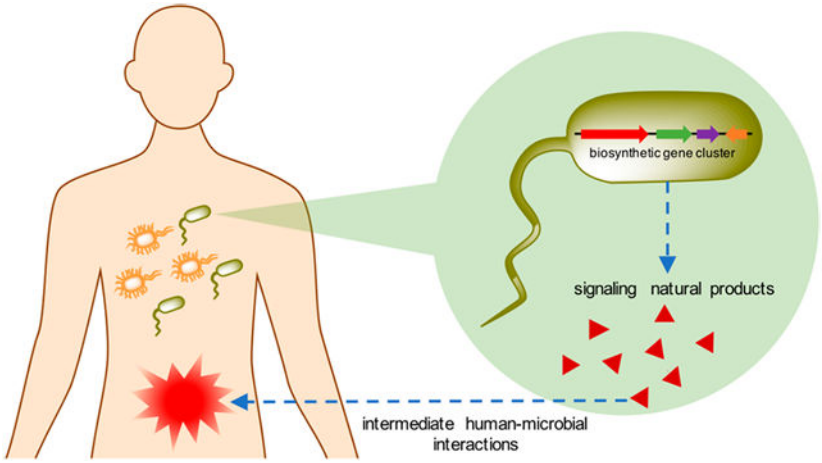
Abstract

Natural products from microorganisms are important small molecules that play roles in various biological processes like cellular growth, motility, nutrient acquisition, stress response, biofilm formation, and defense. It is hypothesized that pathogens exploit these molecules to regulate virulence and persistence during infections. Here, we present selected examples of signaling natural products from human pathogenic bacteria that use these metabolites to gain a competitive advantage. Targeting these signaling systems provides novel strategies to antimicrobial treatments.

Graphical Abstract



Keywords

secondary metabolites; polyketides; nonribosomal peptides; siderophores; virulence; pathogenesis; therapeutic targets

The human body is inhabited by trillions of microorganisms of which the majority are commensal and harmless, yet some are pathogenic and can cause numerous infectious diseases.[1,2] Despite the correlation between human microbiome and disease, the underlying

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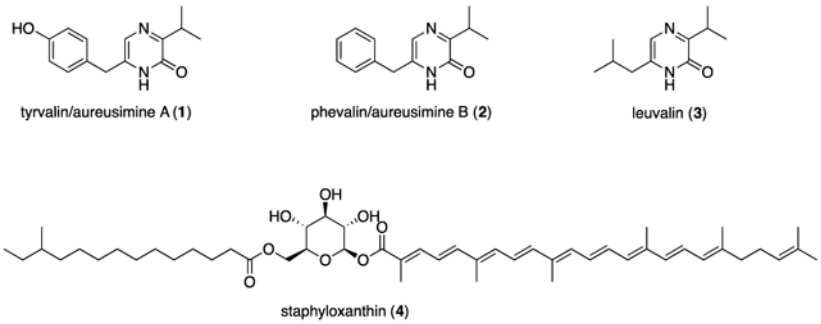
molecular mechanisms have not yet been fully elucidated.[3] Natural products are considered to be signaling instruments of communications between microbe–host and microbe–microbe.[4,5] Examples of these interactions include virulence, biofilm formation, immune modulation, host colonization, nutrient acquisition, and stress response.[6-12] Identifying and characterizing such signaling natural products from the human microbiota might enhance the current understanding of the communications and assist in the development of more effective strategies against human diseases. While great amounts of natural products have been isolated from human microbiota,[13] we predominantly focus on human pathogenic bacteria. Here, we review selected examples of natural products isolated from major human pathogenic bacteria that inhabit the skin, oral and respiratory tracts, and gastrointestinal tract or are found throughout the human body, with specific interests in their structures, bioactivities, and biosynthesis if available. Some important natural products including short-chain fatty acids and ribosomally synthesized and post-translationally modified peptides (RiPPs) are not included since they have been recently reviewed.[14,15]

PATHOGENIC BACTERIA RESIDING ON SKIN

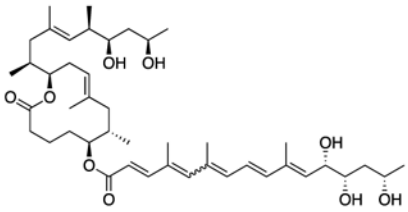
Staphylococcus aureus is classified as Gram-positive skin pathogen commonly causing hospital- and community-acquired infectious diseases such as abscesses, bacteremia, and endocarditis.[16] The emergence of antibiotic resistant *S. aureus*, particularly methicillin resistant (MRSA) strains, is a worldwide problem that needs efforts to develop novel treatment strategies. A dimodular nonribosomal peptide synthetase (NRPS, AusA/PznA) that is conserved across *S. aureus* and other skin-associated Staphylococci is responsible for producing three pyrazinone natural products named tyrvalin/aureusimine A (**1**), phevalin/aureusimine B (**2**), and leuvalin (**3**).[17,18] Their bioactivities were initially related to virulence factor gene expression in *S. aureus*.[18] However, it was later found that the observed gene expression profile actually resulted from an inadvertent mutation in the *sae* two-component sensor kinase gene *saeS*, a known regulator of virulence factor expression.[19,20] Although the biological roles of these compounds remain unknown, recent findings suggested that aureusimines may direct a metabolic switch regulating electron transfer and redox signaling, [20] and aureusimine B was found to be overproduced in *S. aureus* biofilm.[21] It is notable that subsequent research on homologous dimodular NRPSs from gut bacteria produced the same products; however, these products were proposed to be shunt metabolites, and their precursors, the dipeptide aldehydes, were hypothesized to be the active form of these NRPS products that function as protease inhibitors.[6]

Another well-known signaling secondary metabolite of *S. aureus* is staphyloxanthin (**4**), an orange carotenoid pigment considered as its eponymous feature.[22] This pigment is not required for the survival of *S. aureus* but acts as a virulence factor through antioxidant activity with its conjugated double bonds to scavenge free radicals.[23] It was observed that the staphyloxanthin gene deletion strain was more sensitive to reactive oxygen species killing from host neutrophils and was less pathogenic in a mouse subcutaneous abscess model.[24] Targeting staphyloxanthin biosynthesis was then explored to offer novel leads for anti-MRSA infectious drugs. The first biosynthetic step for staphyloxanthin is condensation of two farnesyl diphosphates to generate dehydrosqualene, which is the same for cholesterol biosynthesis. Thus, a known cholesterol biosynthesis inhibitor, BPH-652, was found to

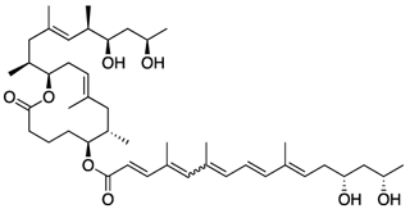
block staphyloxanthin biosynthesis, leading to nonpigmented bacteria and hence more sensitivity to innate immune clearance. This result therefore indicates proof of principle for an antivirulence strategy against *S. aureus.*[25]



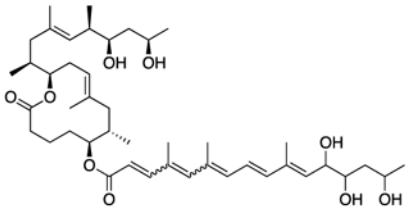
Mycobacterium ulcerans is a Gram-positive human skin pathogen that causes Buruli ulcer, which is characterized by skin ulcers and necrotic cutaneous lesions.[26] Although it had been known for decades that a particular toxin from *M. ulcerans* was related to Buruli ulcer, the specific toxin was not identified until two polyketide-derived macrolides from acetone soluble *M. ulcerans* lipid extracts were isolated.[27] The toxins were mycolactones A (**5**) and B (**6**), which are biosynthesized by giant polyketide synthases (PKSs) encoded by a 174 kb plasmid named pMUM001 in *M. ulcerans*.[28] Mycolactones C (**7**) and D (**8**) were also identified from other clinical isolates of *M. ulcerans*, demonstrating the heterogeneity of these compounds.[29] Various *in vitro* and *in vivo* studies revealed that mycolactones have a crucial part in the pathogenesis of Buruli ulcer, exhibiting cytotoxic, immunosuppressive, and analgesic properties.[30-32] Several molecular targets of mycolactones have been characterized, including Wiskott-Aldrich syndrome protein (WASP) and neuronal Wiskott-Aldrich syndrome protein (N-WASP), Sec61 translocon, type 2 angiotensin II receptors (AT_2Rs), and mechanistic Target of Rapamycin (mTOR), explaining the tissue necrosis, paucity of immune response, and painlessness during the process of Buruli ulcer.[33-36]



mycobactone A (**5**): $\Delta^{4',5'}$=*Z*
mycobactone B (**6**): $\Delta^{4',5'}$=*E*



mycolactone C (**7**)



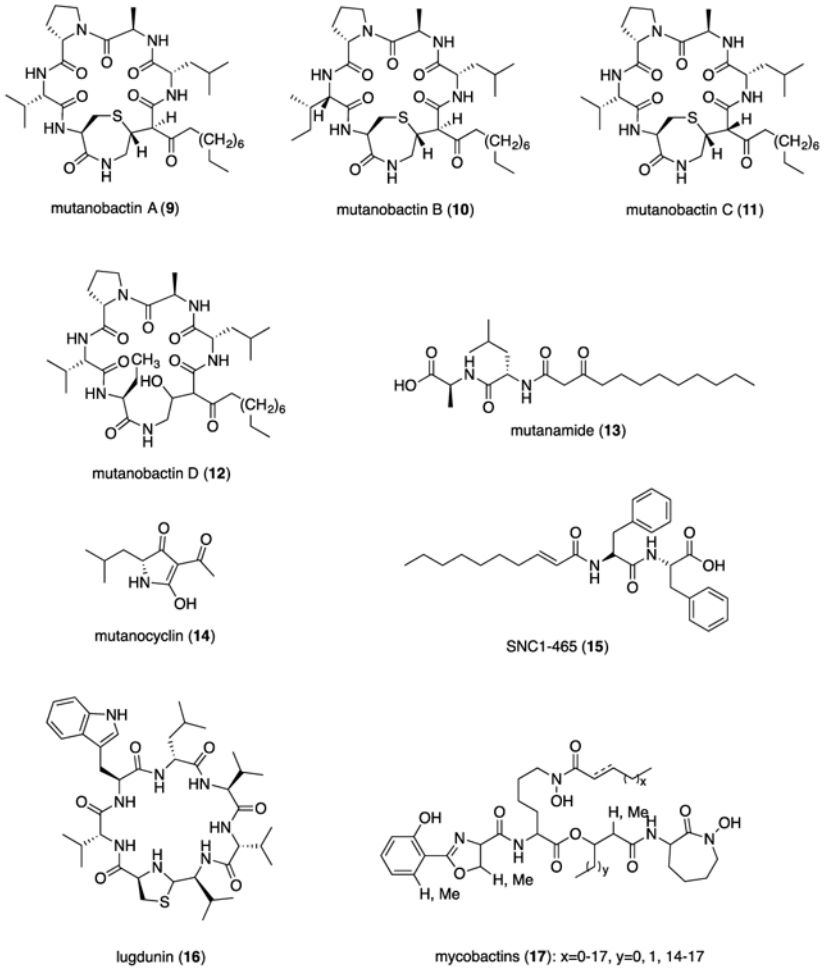
mycolactone D (**8**)

PATHOGENIC BACTERIA RESIDING IN ORAL AND RESPIRATORY TRACTS

Streptococcus mutans is a Gram-positive human commensal and pathogen that has been classified as a primary causative agent in dental cavies.[37] The initial investigation of a hybrid NRPS/PKS containing gene cluster in *S. mutans* UA159 yielded mutanobactin A (**9**) and three of its analogues, mutanobactins B–D (**10–12**).[38,39] Subsequently, systematic comparative metabolomics analysis utilizing the wild-type and mutanobactin gene deletion mutant and precursor feedings afforded 58 metabolites, 13 of which were structurally characterized by detailed MS/MS and isotopically labeled precursor feeding experiments.[40] In addition, a premature product, mutanamide (**13**), was also identified.[40] The bioactivity assessment of mutanobactins revealed that mutanobactins A and B and mutanamide can blunt hyphal generation of the oral-pathogenic fungus *Candida albicans*, while mutanobactins A, B, and D can also perturb the biofilm generation of *C. albicans*.[40] In addition, mutanobactins A and B and mutanamide were subjected to immunomodulatory assays by using the RAW264.7 macrophage cell line. Mutanobactin B was shown to upregulate the pro-inflammatory cytokines like IL-6 and IL-12 in RAW264.7 cells.[40] These results represent a good example of signaling natural products playing dual roles in communications between microbe–microbe and microbe–host interactions. Very recently, *S. mutans* UA159 was validated to be a good heterologous host for the expression of biosynthetic gene clusters (BGCs) from anaerobic bacteria, and the successful activation of BGC1 and BGC4 from human oral bacteria *S. mutans* 35 and *S. mutans* NMT 4863 led to the discovery of mutanocyclin (**14**) and SNC1-465 (**15**), respectively.[41] Mutanocyclin was a tetramic acid biosynthesized by NRPS/PKS, and it was also detected from fermentations of *S. mutans* 35, B30, B409, and B608, suggesting that mutanocyclin is the true product of BGC1 in *S. mutans* isolates. Although no antibacterial activities were detected, mutanocyclin was found to have significant antiinfiltration activity against leukocytes in $CD45^+$ cells.[41] The (2*E*)-decenoyl dipeptide SNC1-465 was also biosynthesized by NRPS/

PKS, but it was proposed to be either an assembly line derailment product or a side product cleaved from the preproduct.[41] The biological role of SNC1-465 remains unknown.

Staphylococcus lugdunensis is a Gram-positive human nasal commensal and pathogen that is associated with osteoarticular infections, foreign-body-associated infections, bacteremia, and endocarditis.[42] *S. lugdunensis* was reported to produce an antibiotic, lugdunin (**16**), that is a nonribosomal cyclic peptide featuring a thiazolidine ring.[10] Lugdunin displayed a broad antimicrobial spectrum against Gram-positive bacteria including *S. aureus* and was found to act as a signaling molecule to prevent *S. aureus* colonization.[10] A further molecular mechanism study revealed that lugdunin also had an immune modulatory activity through upregulating the expression of cytokines such as LL-37 and CXCL8 in epithelial cells to enhance the immune response for effective *S. aureus* clearance.[43]



Mycobacterium tuberculosis is a Gram-positive human pathogenic bacterium that causes tuberculosis. Multidrug resistant (MDR) strains of *M. tuberculosis* have been identified, which represent one of the major threats in infectious diseases.[44] As treatments for MDR tuberculosis are limited, *M. tuberculosis* virulence factors may represent potent options for new drug development. *M. tuberculosis* was reported to produce the NRP-PK mycobactin siderophores (**17**), which are biosynthesized by two genetic loci, mbt-1 (*mbtA-J*) responsible

for mycobactin scaffold assembly and mbt-2 (*mbtK-N*) responsible for the lipid side-chain formation.[45,46] The *mbtB* deletion strain showed attenuated growth in THP-1 cells, while the *mbtE* mutant showed a colony morphology change and also had growth defects in liquid fermentation and macrophages, suggesting mycobactins have an important role in the survival and virulence of *M. tuberculosis*.[47,48] The mycobactin siderophore biosynthesis thus has provided attractive antituberculosis targets, and inhibitors of MbtA, MbtI, and MbtM have been investigated.[49-52] In particular, 5′-*O*-[*N*-(salicyl)sulfamoyl]adenosine (Sal-AMS) exhibited promising inhibitory activity toward the adenylation protein MbtA, acting as a reaction intermediate mimic.[49] *In vitro* studies confirmed Sal-AMS inhibited *M. tuberculosis* growth when iron was limited, while *in vivo* Sal-AMS also inhibited *M. tuberculosis* growth significantly in mouse lungs but showed poor oral bioavailability and clearance rate.[53] Further optimization efforts to enhance pharmacokenetic parameters are ongoing.[54] The *M. tuberculosis* genome harbors another NRPS-encoding gene cluster (*Rv0096-0101*), which is responsible for a putative isonitrile lipopeptide (INLP) production.[55] The *Rv0096-0101* gene cluster was shown to be critical for the *in vivo* survival and virulence of *M. tuberculosis*.[56,57] The expression of this gene cluster was found to be highly upregulated under biofilm formation, albeit the chemical structure of the associated INLP remains elusive.[58]

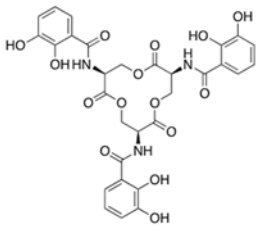
PATHOGENIC BACTERIA RESIDING IN THE GASTROINTESTINAL TRACT

Escherichia coli is a Gram-negative bacterium that usually inhabits the human lower intestine. It commonly acts as a commensal but can be the causative pathogen for diarrheal diseases and extraintestinal infections.[59] *E. coli* has evolved to produce siderophores to acquire iron from a low concentration microenvironment, which is common to many other Gram-negative enteric bacteria like *Salmonella typhimurium, Salmonella enterica,* and *Klebsiella pneumoniae*.[60] Four types of siderophores have been reported from various *E. coli* strains: enterobactin (**18**), salmochelin (**19**), yersiniabactin (**20**), and aerobactin (**21**).[61] Enterobactin is a 2,3-dihydroxybenzoylserine trilactone biosynthesized by a dimodular NRPS from 2,3-dihydroxybenzoic acid and serine. Although enterobactin has a high efficiency for iron capture, hosts fight back by producing siderocalin, a small protein that binds enterobactin and stops its iron uptake. Pathogenic bacteria respond to the threat by generating salmochelin, a glycosylated derivative of enter-obactin. Such a structure modification of enterobactin prevents capture by siderocalin and restores efficient uptake of iron by pathogens.[62] Yersiniabactin is an NRP-PK hybrid natural product assembled from salicylate, three cysteines, a malonyl group, and three methyl groups by an NRPS/PKS complex.[63,64] The receptor of yersiniabactin was identified to develop a vaccine of pyelonephritis in an *E. coli*-caused urinary tract infectious mouse model.[65] Aerobactin is a citryl-hydroxamate siderophore synthesized by the condensation of two oxidized lysines with citric acid, which is widely distributed in pathogenic Gram-negative bacteria to promote iron uptake.[66] It is notable that knocking out only aerobactin resulted in a significant attenuation of virulence in a hypervirulent strain of *Klebsiella pneumoniae*, a life-threatening infectious agent, suggesting the critical role of this metabolite during infection.[66]

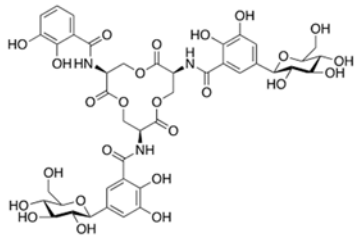
Some *E. coli* strains can produce a genotoxin, colibactin, which has been associated with the pathogenesis of colorectal cancer in human hosts.[67-69] The biosynthesis of colibactin is

linked to a 54 kb NRPS/PKS hybrid gene cluster (*clb*),[70] but the structure characterization of colibactin has been blocked by both the complexity of the *clb* gene cluster and the instability of the genotoxic metabolite.[71-81] Current extensive work from multiple independent groups suggested that the overall genotoxic effect of the *clb* gene cluster may arise from a mixture of metabolites with different activities.[82-85] At least two activities, the DNA cross-linking and double-strand break (DSB) activities, have been associated with different metabolites (colibactin (**22**) and colibactin-645 (**23**), respectively) produced by promiscuous enzymes encoded by *clb*.[86,87]

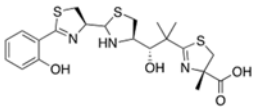
Klebsiella oxytoca are Gram-negative human gut bacteria that cause antibiotic-associated hemorrhagic colitis (AAHC), a right-sided segmental colitis featured by bloody diarrhea and severe cramps.[88] The initial investigation of the molecular mechanism for the pathogenesis of colitis caused by *K. oxytoca* revealed a pyrrolobenzodiazepine natural product tilivalline (**24**).[89] Tilivalline was observed to induce human cell apoptosis and block epithelial barrier function, which resulted in mucosal damage in AAHC.[89] A recent study of the biosynthesis of tilivalline demonstrated that its NRPS first generates tilimycin (**25**), followed by a nonenzymatic reaction with indole affording tilivalline. It has also been observed that tilimycin is spontaneously converted to another product culdesacin (**26**).[90,91] While culdesacin demonstrated no obvious bioactivity, tilimycin showed a higher cytotoxic activity to human cells than tilivalline. The detailed mode of action study of these two toxins indicated that tilimycin acts as a genotoxin to cause DNA strand breakage while tilivalline binds tubulin and stabilizes microtubules leading to mitotic arrest, contributing collectively in the pathogenicity of colitis.[92]



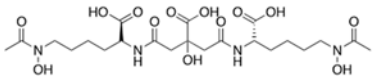
enterobactin (**18**)



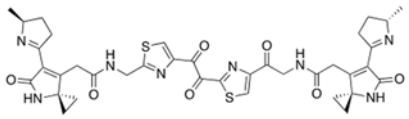
salmochelin S4 (**19**)



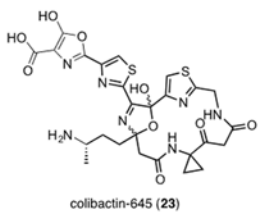
yersiniabactin (**20**)



aerobactin (**21**)

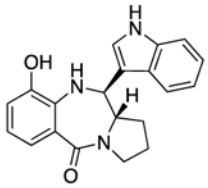


colibactin (**22**)

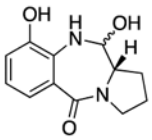


colibactin-645 (**23**)

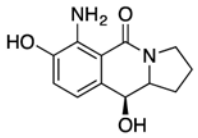
Bacillus cereus are Gram-positive bacteria, and some strains are harmful to humans, causing foodborne illness.[93] Cereulide (**27**) was reported to be the causative toxin of the emetic foodborne diseases originating from *B. cereus*.[94] It is a depsipeptide containing six α-amino acids and six α-hydroxy acids that are biosynthesized by an NRPS (Ces).[95] Cereulide caused mitochondrial swelling of HEp-2 cells and consequently cell death,[94] and the toxic effects of cereulide were proposed to be due to its ionophoretic properties.[96]



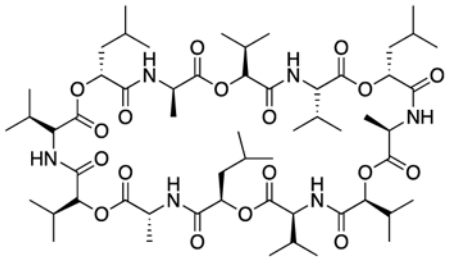
tilivalline (**24**)



tilimycin (**25**)



culdesacin (**26**)

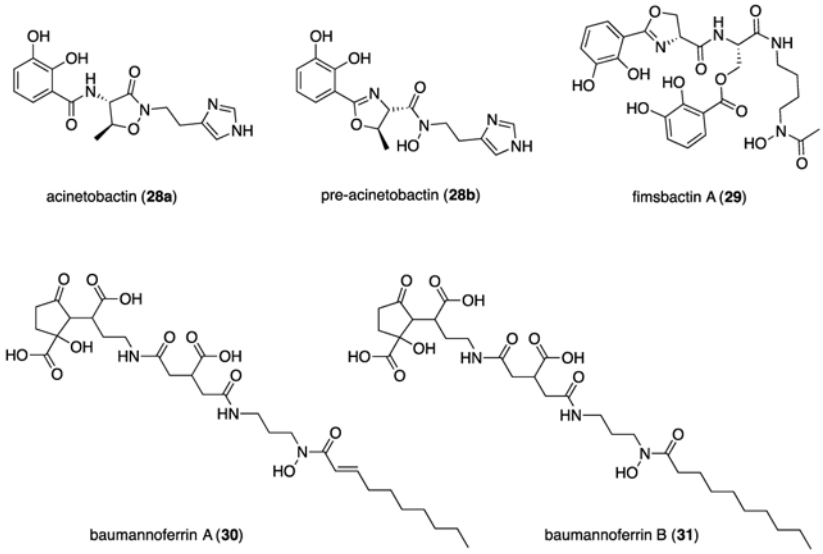


cereulide (**27**)

PATHOGENIC BACTERIA RESIDING THROUGHOUT THE HUMAN BODY

Acinetobacter baumannii are Gram-negative human pathogenic bacteria that cause hospital- and community-acquired infections in the skin, respiratory tract, blood, urinary tract, and other soft tissues.[97,98] The emergence of multidrug resistant *A. baumannii* is a high threat to human health, and it lacks efficient treatments. To successfully cause infections in a host, *A. baumannii* utilizes siderophores to compete for iron from the host. Three types of siderophores have been observed from clinical isolates of *A. baumannii*: acinetobactin, fimsbactin, and baumannoferrin.[99] Acinetobactin (**28**) is a catechol-hydroxamate siderophore and is assembled from *N*-hydroxyhistamine, L-threonine, and 2,3-dihydroxybenzoic acid by an NRPS assembly line.[100,101] Acinetobactin was initially generated in preacinetobactin with an oxazoline group (**28b**) and rapidly isomerizes nonenzymatically into isoxazolidinone acinetobactin (**28a**) under basic conditions.[102] This pH-triggered siderophore swapping enables its iron uptake over a broad pH range during an infection. Bioinformatic analysis of 50 clinical isolates indicated that acinetobactin was highly conserved in most *A. baumannii* isolates.[103] Fimsbactin A (**29**) to Fimsbactin F are catechol-hydroxamate siderophores and only distributed in the *A. baumannii* ATCC 17978 clinical isolate.[103,104] According to its genome information, a 26 kb gene cluster containing NRPS genes was found to be responsible for biosynthesis, secretion, and utilization of

fimsbactins.[104] Baumannoferrins A (**30**) and B (**31**) contain only hydroxamates and were isolated from *A. baumannii* AYE that does not produce acinetobactin.[105] Baumannoferrins A and B are derivatized from citrate, 1,3-diaminopropane, 2,4-diaminobutyrate, decenoic acid, and α-ketoglutarate. The discovery of different siderophores from different *A. baumannii* strains suggests the critical role of siderophores for this pathogen, although different siderophore-mediated uptake systems could be used to fulfill the need.



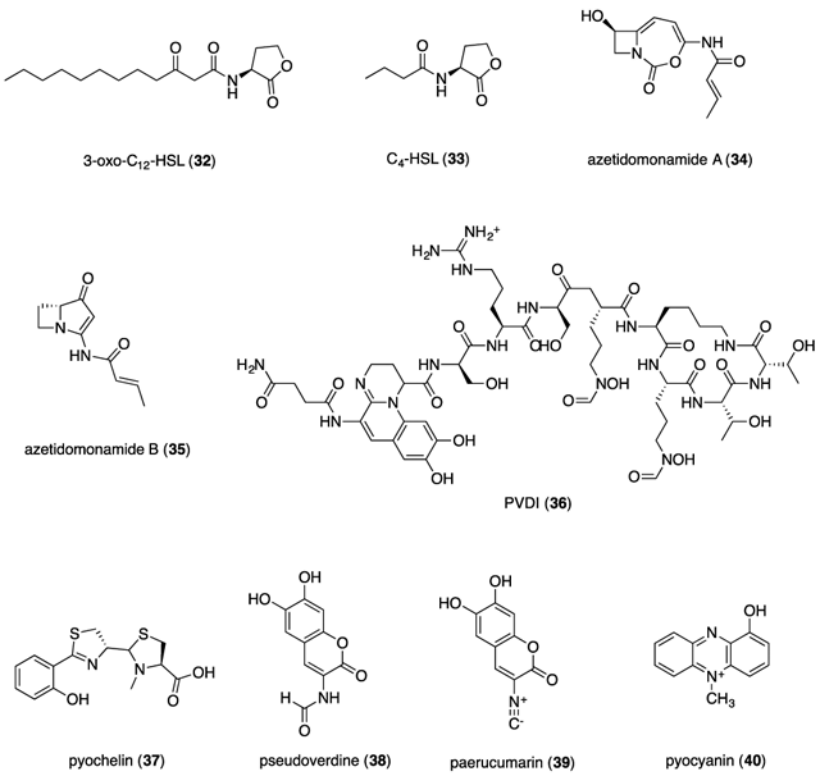
Pseudomonas aeruginosa is a Gram-negative opportunistic human pathogen that can infect virtually all tissues.[106,107] *P. aeruginosa* possesses multiple signaling networks that coordinately regulate virulence and persistence during infections, making it a major threat to human health.[108] *N*-Acyl homoserine lactones (AHLs) are quorum sensing signaling molecules biosynthesized from *S*-adenosylmethionine by many Gram-negative bacteria to control gene expression.[5] *P. aeruginosa* was observed to have two AHL systems: Las and Rhl systems. The former one is regulated by *N*-3-oxododecanoyl homoserine lactone (3-oxo-C_{12}-HSL, **32**),[109] which controls various virulence gene expressions involved in acute infection and host cell damage.[110] The latter one is regulated by *N*-butanoyl homoserine lactone (C_4-HSL, **33**),[111] which negatively controls the expression of type III secretion regulon.[112] An *in vivo* study showed that AHL system deficiencies attributed to a decrease in infection severity.[113] In addition, quorum sensing has been found to regulate other secondary metabolite biosyntheses in *P. aeruginosa.* For example, recent studies from two independent groups identified rare azetidine-containing alkaloids, azetidomonamides A (**34**) and B (**35**),[114,115] which are biosynthesized from a conserved NRPS pathway regulated by quorum sensing. No antibacterial and cytotoxic activities were observed, while azetidomonamide gene deletion strains displayed rapid virulence in a *Galleria mellonella* model, suggesting a host adaption function for these metabolites.[114]

P. aeruginosa also utilizes siderophores for iron acquisition under iron limiting conditions. It produces two kinds of peptide siderophores biosynthesized by NRPSs: pyoverdines and pyochelin. The structures of pyoverdines (PVDs) contain a dihydroquinoline chromophore, a 6–12 amino acids peptide, and a side chain that varies in succinate, malate, α-ketoglutarate,

or their amide derivatives.[116] More than 60 PVDs have been determined from different *Pseudomonas*, while *P. aeruginosa* was found to produce PVDI (**36**), PVDII, and PVDIII, which differ by the peptide chain.[117] The biogenesis, maturation, and transport of PVDs were linked to the *pvd* locus with divergence across different strains, indicating a strain-specific structure diversity of PVDs.[118-121] Pyochelin (**37**) is biosynthesized from a salicylate, a hydroxy acid, and two cysteines by an NRPS-encoding gene cluster *pch*.[122] Its iron chelation ability is much lower than that of pyoverdine.[123] It was observed that pyochelin was first produced and then switched to pyoverdine only if iron concentration became very low.[123] Both pyoverdine and pyochelin are essential for survival and virulence gene expression for infections in immunosuppressed mice models.[124] Pyoverdine was also found to act as a key inhibitory molecule for the biofilm formation of *Aspergillus fumigatus* that resides in the same body niche, suggesting signaling interactions between different kingdoms.[125]

The *P. aeruginosa* genome contains a *pvc* gene cluster that was initially linked to pseudoverdine (**38**) production, a fluorescent bicyclic compound similar to the pyoverdine chromophore.[126] Due to the similarity between PvcA and isonitrile synthases, further investigation of the *pvc* gene cluster revealed a new metabolite paerucumarin (**39**), an isonitrile functionalized cumarin.[127] It was found that *pvc* operon can enhance the expression of the chaperone/usher pathway (cup) genes related to biofilm formation and the iron-controlled genes, and this regulation was mediated through paerucumarin.[128,129] Besides, the biosynthetic intermediate, isonitrile-functionalized tyrosine was observed to modulate swarming motility and quorum sensing in *P. aeruginosa*.[130]

P. aeruginosa secretes another family of virulence factor, phenazines. Pyocyanin (**40**) is the most well studied one and is related to its blue-green color feature.[131] Pyocyanin is a redoxactive tricyclic zwitterion and contributes to both acute and chronic infections since it inhibits lymphocyte proliferation,[132] damages epithelial cells,[133] and inactivates protease inhibitors to cause tissue damage.[134] The biosynthesis of pyocyanin involves *phz1* and *phz2* that synthesize phenazine-1-carboxylic acid (PCA) and *phzM* and *phzS* that convert PCA to pyocyanin.[135,136]



CONCLUSIONS

Chemical signaling has been known to play an important role in bacterial infection and pathogenesis, but the underlying molecular mechanism and the responsible specialized bacterial metabolites often remain elusive. Recent technological advances in genome sequencing, bioinformatics, genome editing, synthetic biology, and analytical chemistry have promoted the identification and characterization of many new bioactive natural products from pathogenic bacteria. Since the emergence of drug resistant pathogens arises faster over the development of new antibiotics, blocking these signaling systems in the pathogens is expected to overcome the existing resistant mechanisms and provide new strategies for treatment. In addition, as many of these specialized metabolites are not essential to *in vitro* survival but have a critical role during *in vivo* infection, they may represent new antimicrobial targets for which the pathogen has less of a chance of developing drug resistance. We thus expect to see a further development in this field to effectively combat bacterial infections, in particular toward strains that are multidrug resistant.

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