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Acyl-histidines: New *N*-acyl amides from *Legionella pneumophila*

Thomas Tørring[a], Stephanie R. Shames[b], Wooyoung Cho [c,d], Craig R. Roy [b] and Jason M. Crawford*[b,c,d]

Abstract: *Legionella pneumophila*, the causative agent of Legionnaires' disease, is a Gram-negative Gammaproteobacterial pathogen that infects and intracellularly replicates in human macrophages and a variety of protozoa. *L. pneumophila* encodes an orphan biosynthetic gene cluster (BGC) that contains isocyanide-associated biosynthetic genes and is upregulated during infection. Because isocyanide-functionalized metabolites are known to harbor invertebrate innate immunosuppressive activities in bacterial pathogen-insect interactions, we used pathway-targeted molecular networking and tetrazine-based chemoseletive ligation chemistry to characterize the metabolites from the orphan pathway in *L. pneumophila.* We also assessed their intracellular growth contributions in an amoeba and in murine bone marrow derived macrophages. Unexpectedly, two distinct groups of aromatic amino acid-derived metabolites were identified from the pathway, including a known tyrosine-derived isocyanide and a family of new *N*-acyl-L-histidine metabolites.

Introduction

Most commonly referred to as an accidental human pathogen, *Legionella pneumophila* thrives in both anthropogenic and environmental water systems where it is found replicating as an intracellular pathogen in protozoa such as amoebae.[1] *L. pneumophila* produces a wide variety of often functionally redundant virulence factors enabling it to infect a spectrum of protozoa. This host diversity has facilitated its ability to infect and intracellularly replicate in human macrophages.[1] Human infection results in either a mild influenza-like disease called Pontiac fever or the much more severe pneumomia termed Legionellosis (Legionnaires' disease). In the United States alone, for example, about 5000 individuals are diagnosed with Legionellosis every year, roughly half of which required admission to an intensive care unit, and with a total mortality rate of approximately 9%.[2] Infection normally occurs through inhalation of contaminated aerosols from hot tubs, hot water tanks, or cooling towers.

In macrophages, *L. pneumophila* is taken up by phagocytosis, but the bacterium employs a type 4 secretion system known as Dot/Icm (defect in organelle trafficking/ intracellular multiplication) to prevent the normal fusion with lysosomes and create a specialized *Legionella*-containing vacuole (LCV). *L. pneumophila* is predicted to employ a staggering number of more than 300 effector proteins during infection.[3,4] The remaining effector proteins are hypothesized to stem from the broad range of hosts infected by *L. pneumophila*. This is also supported by a high degree of functional redundancy where single gene deletion mutants rarely result in significant intracellular growth defects.[3,4] While the protein effectors have been intensely studied over the past decade, few secondary metabolites have been thoroughly investigated in *Legionella* strains. A recent survey described several predicted biosynthetic gene clusters (BGCs) encoding polyketide synthases (PKSs) and nonribosomal peptide synthetases (NRPSs) spread among various *Legionella spp*. As part of that study, legionellol, a polyketide-derived surfactant that affected sliding mobility was identified.[5] Other secondary metabolites from *Legionella* include the siderophore legiobactin[6] and the quorum sensing auto-inducer LAI-1.[7] We were intrigued by a BGC in *L. pneumophila* containing the isocyanide-associated genes *isnA* and *isnB* because previous work has demonstrated that isocyanide-containing secondary metabolites dramatically affect innate immunosuppression in an invertebrate animal infection model, *Galleria mellonella*.[8] Given the fact that the *isnA* and *isnB* genes are clustered in various human pathogens, we aimed to elucidate the structures of the secondary metabolites encoded by this BGC using heterologous expression, a chemoselective tetrazine probe, gene deletion in wild-type *L. pneumophila*, and intracellular growth in both amoebae and macrophages. Unexpectedly, the BGC produced two distinct groups of secondary metabolites derived from aromatic amino acid substrates, one of which constitutes the first metabolite members of histidine-derived *N*-acyl amides. *N*-acyl-amides are well known in lipidomics and chemical signaling where the acyl-groups are often appended via an acyl-transferase.[9] Interestingly, the biosynthesis of *N*-acyl-histidines was not derived from an acyl-transferase, but rather an ATP-grasp enzyme.

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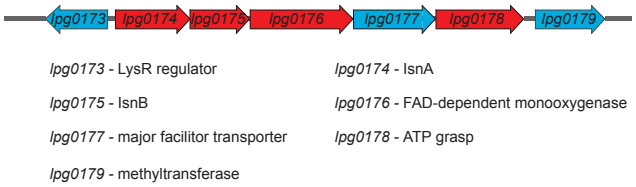

Figure 1. Acyl-histidine and isocyanide biosynthetic gene cluster. Biosynthetic genes are colored red. Regulatory and transport genes are colored blue.

Results and Discussion

***L. pneumophila* encodes an "orphan" isocyanide biosynthetic gene cluster.**

Homologs of isocyanide biosynthetic genes, *isnA* (*lpg0174*) and *isnB* (*lpg0175*),[10-12] in *L. pneumophila* (subsp. *pneumophila* strain Philadelphia 1)[13] are clustered on a predicted five-gene operon (*lpg0174-lpg0178*, Figure 1). The operon is flanked by a putative *lysR*-family transcriptional regulator (*lpg0173*), which is likely involved in its regulation, and a predicted O-methyltranferase (*lpg0179*). *L. pneumophila* strain Corby encodes an orthologous pathway, and a transposon mutant in the O-methyltransferase (*lpc0263*) was reported to lead to a defect in the strain's natural lysozomal avoidance phenotype, although the mutant phenotype was not verified by genetic complementation.[14] In addition to the isocyanide biosynthetic genes in the operon, it encodes homologs of a FAD-dependent oxidoreductase (*lpg0176*), a major facilitator superfamily (MFS) efflux transporter (*lpg0177*), and an ATP-grasp protein (*lpg0178*). Interestingly, the gene cluster is up-regulated at the transcriptional level during intracellular bacterial growth in human macrophages,[15] suggesting a potential role in infection.

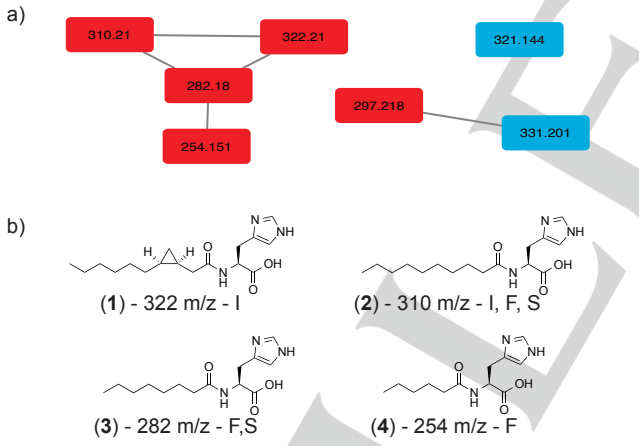

Figure 2. Pathway-targeted molecular networking. a) Red color nodes mark molecular ions observed in both *E. coli* expressing the pathway and *L. pneumophila*. Blue color is associated with ions that could only be observed in the *E. coli* strain. Nodes are interconnected by tandem-MS relationships. Metabolomic analysis was conducted in positive ion mode. b) New *N*-acyl-L-histidine metabolites (**1-4**) identified and characterized in this study. I, isolated; F, confirmed by free fatty acid feeding studies; and S, confirmed by synthesis.

Pathway-targeted analysis pinpoints new acyl-histidines in *L. pneumophila*.

We first cloned the operon *lpg0174-lpg0178* (LegA) and the O-methyltransferase *lpg0179* (LegB) downstream of two independent isopropyl-β-D-1-thiogalactopyranoside (IPTG)-inducible T7 phage promoters in pETDuet (pLegAB, Supporting Figure 1, Supporting Table 1). The construct was transformed into *E. coli* BAP-1[16] for heterologous expression and metabolite analysis. The cultures were grown in lysogeny broth (LB) supplemented with ampicillin under aerobic conditions and at a temperature of 30°C. n-Butanol extracts of 5 mL cultures of induced *E. coli* BAP-1 carrying either pLegAB or its empty vector control were analyzed by comparative metabolomics and a molecular networking technique,[17] "pathway-targeted" molecular networking.[18,19] Molecular networking allows for metabolite connections based on tandem MS fragmentation relationships, and pathway-targeted molecular networking maps only the ions dependent on the presence of the pathway. Our pathway-targeted analysis of the *E. coli* strain in positive ion detection mode resulted in one distinct molecular family that was also detected in wild-type *L. pneumophila* (Figure 2, Supporting Figure 2). Two abundant metabolite ions in this molecular family, 322.2130 and 310.2119, were targeted for isolation and structural elucidation. Their high-resolution masses were consistent with the molecular formulas, $C_{17}H_{27}N_3O_3$ (calc, $M+H^+$, 322.2125) and $C_{16}H_{27}N_3O_3$ (calc, $M+H^+$, 310.2125), respectively. The metabolites were extracted from a large-scale culture (6 L) with n-butanol and were purified by a series of normal- and reversed-phase chromatographic steps guided by mass spectrometry, resulting in the isolation of new *N*-acyl-L-histidine metabolites **1** (<1 mg) and **2** (2 mg). Their planar structures were determined through one- (^{1}H) and two-dimensional (gCOSY, gHSQCAD, and gHMBCAD) NMR experiments (Supporting Tables 2-3). The acyl groups of these major metabolites featured a straight-chain decanoyl-moiety (**2**) and a rare β,γ-cyclopropanated decanoyl-moiety (**1**), supporting a biosynthetic preference for C10-chain-lengths.

The absolute configurations of acyl-histidines **1** and **2** were supported by chemical degradations, ^{1}H-NMR resonance signatures, and bacterial genetics. Acid hydrolysis of **1** and **2** and subsequent Marfey's analysis[20] established S-configurations in their histidine moieties (Supporting Figure 3). This completed the structure of **2** as *N*-decanoyl-L-histidine. The cyclopropane ring system in **1** can be assigned as *cis* based on interpretation of the NMR data and the signature negative chemical shift value of the methylene proton *cis* to the alkyl substituents (Figure 3b).[21,22]

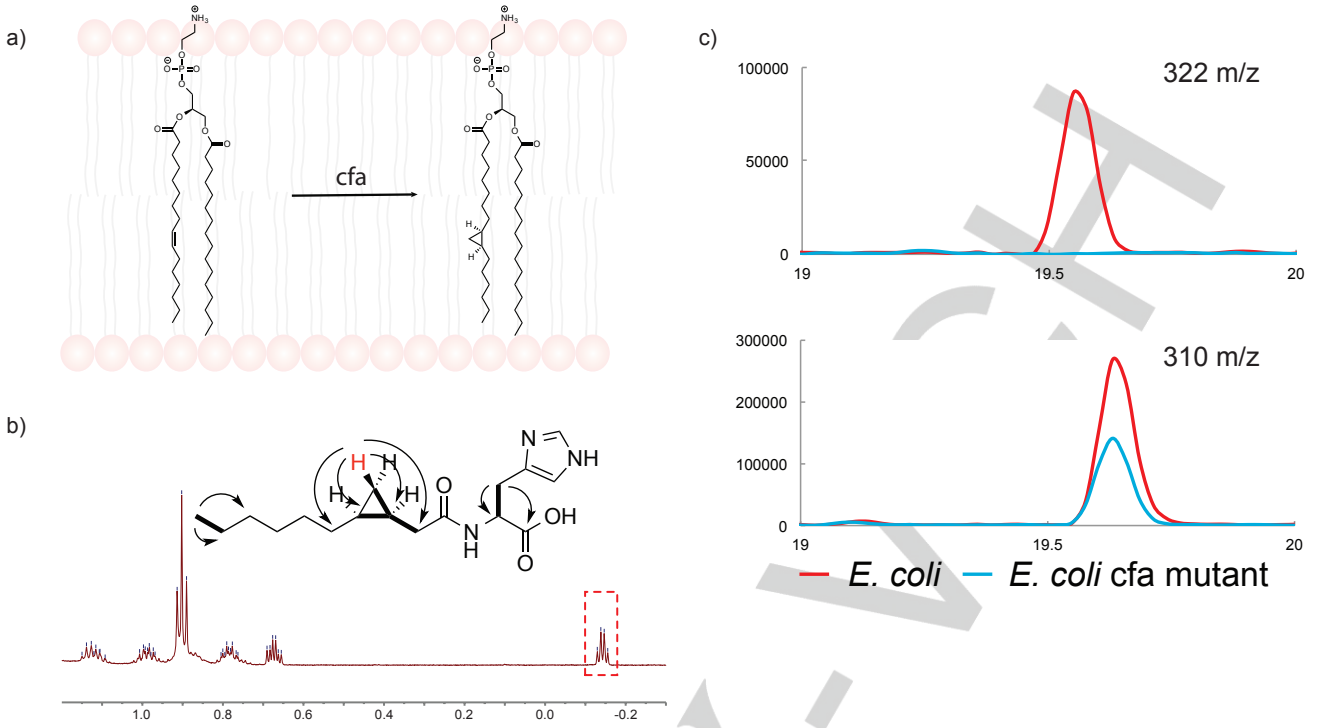

Figure 3. Biosynthesis and stereochemical assignment of cyclopropropanated *N*-acyl-L-histidine **1**. a) Cfa-catalyzed SAM-dependent methylation of cis-unsaturated fatty acid moiety in cyclopropanated phospholipid biosynthesis. b) ^{1}H-NMR spectrum supporting a cis-configuration of the cyclopropane moiety. The characteristic chemical shift value supporting a cis conformation is highlighted in the red box. c) LC-MS extracted ion count (EIC) chromatograms of *E. coli* wild-type (red) and *cfa* mutant (blue) containing the dual acyl-histidine-isocyanide biosynthetic gene cluster.

We hypothesized that this acyl-group was derived from an endogenous cyclopropanated decanoic acid. Cyclopropanation of unsaturated fatty acids in *E. coli* is observed on existing phospholipids. This reaction is carried out by a SAM-dependent methyltransferase, cyclopropane fatty acid synthase (CFAS) (Figure 3a). To determine whether the cyclopropane moiety in **1** originates from CFAS, we introduced the pathway (pLegAB) and pTARA:500[23] (a plasmid with an arabinose-inducible expression system for the T7 phage RNA polymerase) into *E. coli* BW25113 and its corresponding *cfa* mutant strain.[24] n-Butanol extracts from both strains were analyzed using LC-MS. While the production of **2** with a straight-chain fatty acid was only slightly lower in the *cfa* mutant, the production of **1** was completely abolished (Figure 3c). This experiment allowed us to assign the *cfa*-dependent biosynthetic origin of the cyclopropanated acyl-group in **1** and support a 3*S*, 4*S* stereochemical assignment.[25] The gene, *lpg1131* in *L. pneumophila*, encodes a CFAS homolog (protein identity/similarity: 54%/71%), and we therefore find it likely that **1** is produced in a similar manner in *L. pneumophila.* Considering that membrane-associated phospholipids represent the known substrates for CFAS, the cyclopropanated substrate is likely recruited from the phospholipid catabolism which is a typical occurance in cells entering stationary phase,[26] the cyclopropanated substrate is likely recruited from phospholipid catabolism.

The chromatographic behaviors, tandem MS fragmentations, predicted molecular formulas, and pathway-targeted molecular network data for the remaining detectable ions 308.1962 (**5**), 282.1804 (**3**), and 254.1508 (**4**) suggested additional *N*-acyl-L-histidine analogs, featuring decenoyl-, octanoyl-, and hexanoyl-side chains. Synthetic standards of **2**, **3**, and **4** were prepared. The synthetic standards shared identical retention times, high-resolution masses, and fragmentation patterns with their corresponding natural products, confirming structural assignments.

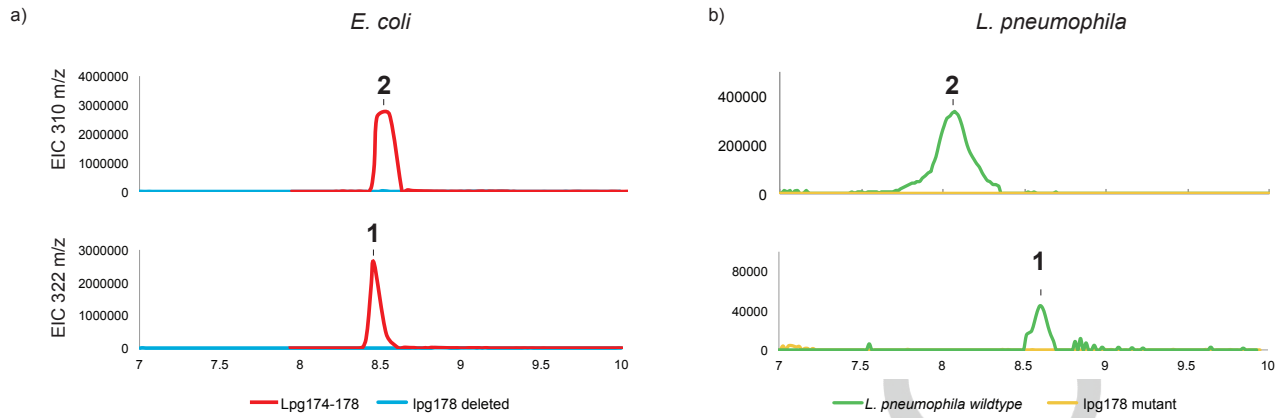

Figure 4. Genetic dependency of *lpg0178* on *N*-acyl-L-histidine biosynthesis. a) Extracted ion chromatograms of the 310 *m/z* and 322 *m/z* from n-butanol extracts of *E. coli* grown in lysogeny broth containing the biosynthetic gene cluster with (red) and without (blue) the gene encoding the ATP-grasp enzyme *lpg0178*. b) Extracted ion chromatograms of the 310 *m/z* and 322 *m/z* from n-butanol extracts of *L. pneumophila* grown in buffered charcoal yeast extract containing the biosynthetic gene cluster with (green) and without (yellow) the gene encoding the ATP-grasp enzyme *lpg0178*. Tandem-MS spectra of the compounds can be viewed in Supporting Figures 15 and 16. Co-injection experiments of **1** from *E. coli* and from *L. pneumophila* support an identical structure (Supporting Figure 6).

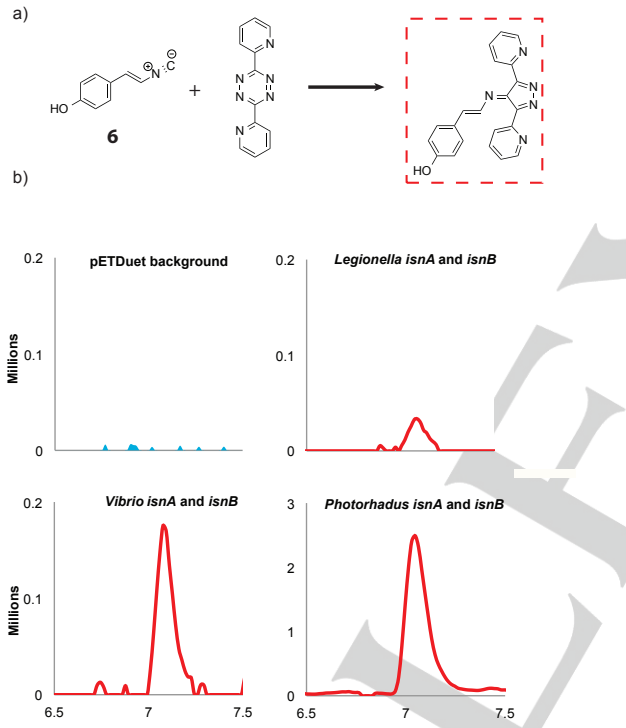

Figure 5. Trapping genetically encoded isocyanide metabolites with a tetrazine probe. a) Chemoselective reaction between an isocyanide (known from *P. luminescence* and *V. cholerae*) and a tetrazine probe. b) EICs of the resulting trapped isocyanide (354.135 m/z) in *E. coli* with a pETDuet negative control background, the dual isocyanide / *N*-acyl-L-histidine biosynthetic gene cluster (*lpg0174-lpg0178*) from *L. pneumophila*, *isnA-isnB* from *V. cholerae*, and *isnA-isnB* from *P. luminescens*.

To determine the genetic dependencies on metabolite production, we constructed individual deletions of every biosynthetic gene in the pathway (Δ*lpg0174*, Δ*lpg0175*, Δ*lpg0176*, Δ*lpg0178*, and Δ*lpg0179*) for heterologous production in *E. coli* BAP-1 (Supporting Figure 4). The ATP-grasp gene (*lpg0178*) was the only gene required for *N*-acyl-L-histidine **1-5** biosynthesis (Figure 4a, Supporting Figure 5). Similarly, we analyzed n-butanol extracts of wild-type *L. pneumophila* and a series of transposon mutants in the pathway covering every gene (*lpg0173* through *lpg0179*). Both **1** and **2** could be observed in wild-type *L. pneumophila* (Figure 4b) and a co-injection of extracts supports an identical stereoconfiguration of **1** (Supporting Figure 6). The metabolites were detected in the *L. pneumophila* transposon mutants (albeit at lower intensities) except for mutations in the ATP-grasp gene, in which they were completely abrogated (see Supporting Figure 7). Mutants in the methyltransferase *lpg0179* located in the gene cluster (not the CFAS homolog) showed production of both **1** and **2** at intensities comparable to wild-type, indicating that *lpg0179* was not involved in the cyclopropanation reaction (see Supporting Figure 8). These experiments validate the function of the ATP-grasp protein and its involvement in *N*-acyl-L-histidine biosynthesis in *L pneumophila*. Based on protein homologies, the ATP-grasp protein (PF13535) likely functions as an ATP-dependent ligase, recruiting free medium-chain-length fatty acids from primary metabolism and linking them to free L-histidine substrates. This biosynthetic strategy is in stark contrast not only to the SAM-derived biosynthesis of acyl-homoserine lactones,[27] but also to the *N*-acyl transferase family (PF13444) responsible for synthesizing *N*-acyl-tyrosines, *N*-acyl-arginines, and commendamide.[28] In further support of this notion, supplementation of the culture medium with free C6-, C8-, and C10-chain length fatty acids led to dramatically enhanced production of their corresponding *N*-acyl-L-histidines (Supporting Figure 9). Unsaturated C10-fatty acids with an α/β-cis- (a known *Pseudomonas* biofilm inhibitor[29]), an α/β-trans-, and a β/γ-cis-double bond were also readily accepted leading to their *N*-decenoyl-L-histidines. Lastly, it is worth noting that L-histidine biosynthesis is up-regulated during intracellular bacterial growth in human macrophages.[15]

A tetrazine probe identifies an isocyanide metabolite

The *N*-acyl-L-histidines described above do not support the presence of isocyanide biosynthetic genes in the gene cluster. To identify isocyanide-functionalized metabolites, we designed a general isocyanide detection scheme using established tetrazine-coupling chemistry. Tetrazines have been shown to chemoselectively react with isocyanides in a [4+1] cycloaddition reaction followed by release of N_2 even in complex cellular enviroments.[30] We found that addition of 3,6-di-2-pyridyl-1,2,4,5-tetrazine and methanol to either M9 medium containing casamino acids or bacterial cultures in the same medium (*Vibrio cholerae* O1 biovar El Tor N16961 was used for method development) could trap a supplemented isocyanide-functionalized molecule (4-methoxyphenyl isocyanide) with lower than 1 μM detection limits even on a simple single quadrupole LC/MS (Supporting Figure 10). This adduct could readily be extracted with n-butanol and identified by its signature UV-visible spectrum and product mass by LC/MS, supporting a general route for isocyanide-functionalized metabolite discovery.

With the LC/MS-based isocyanide-detection scheme, we then screened *E. coli* heterologously expressing the known isocyanide biosynthetic genes (*isnA* and *isnB*) from *Photorhabdus luminescens* and *Vibrio chlolerae.*[8,31] We compared these cultures to *E. coli* harboring the orphan *L. pneumophila* pathway (*lpg0173-lpg0179*). In all cases, only a single tetrazine-isocyanide adduct was detected in positive ion mode, which matched the known isocyanide **6** (Figure 5). With this knowledge, we extracted unreacted cultures with ethyl acetate and we were able to identify free isocyanide **6** in the cultures using negative ion mode mass spectrometry (Supporting Figure 11). This molecule represents a nanomolar-level inhibitor of phenoloxidase, a critical component of invertebrate innate immune systems.[8] While the isocyanide could readily be identified in the heterologous production cultures, **6** was not detected in wild-type *L. pneumophila* under the conditions of our experiment. In wild-type *L. pneumophila*, the isocyanide biosynthetic genes may be functionally "silent" under our cultivation conditions or **6** is further processed to other currently undetectable metabolites. While looking for other potential isocyanide metabolites, we determined that the tetrazine probe formed an adduct with palmitoleic acid (Supporting Figure 12). This off-target reactivity on a common precursor should be generally considered in the various applications of tetrazine probes, particularly in "bioorthogonal" applications. Collectively, our probe-based experiments confirm that *L. pneumophila* has the genetic potential to produce isocyanide products/intermediates and that the single orphan gene cluster encodes not one, but two distinct types of aromatic amino acid-derived natural products, *N*-acyl-L-histidines and the tyrosine-derived isocyanide. The clustered organization of genes suggests that there is a possible benefit to the coordinate transcriptional regulation of these distinct families of natural products in *L. pneumophila*.

Bacterial genetics enabled preliminary interrogation of pathway function

Based on observation of the up-regulation of this gene cluster during intracellular infection, we examined the impact of isocyanide and acyl-histidine production on intracellular growth of *L. pneumophila* in murine bone marrow-derived macrophages (BMDMs) and the natural protozoan host *Acanthamoeba castellanii*. *NLRC4*$^{-/-}$ BMDM, which are permissive to infection with flagellated *L. pneumophila,*[32] and *A. castellanii* were infected with wild-type (SRS43), *lpg0174*::Tn, or *lpg0178*::Tn mutant *L. pneumophila* followed by enumeration of colony forming units (CFUs) recovered over 72 hours. We observed a significant defect in intracellular replication of the *lpg0178*::Tn mutant compared to wild-type *L. pneumophila* in *NLRC4*$^{-/-}$ BMDM (*P < 0.05, **P < 0.01, 13). The *lpg0178*::Tn mutant also exhibited a subtle but significant growth attenuation in *A. castellanii* (*P < 0.05, **P < 0.01, Supporting Figure 13). We subsequently examined the impact of isocyanide production on intracellular replication of *L. pneumophila* and found that the *lpg0174*::Tn mutant could replicate to the same extent as the wild-type strain in *NLRC4*$^{-/-}$ BMDM and *A. castellanii* (Supporting Figure 13). This prompted us to construct clean deletions of *lpg0178* and of the entire *lpg0173-lpg0179* gene locus (Δ*locus*) to verify the phenotype observed for the transposon mutants. However, we were unable to distinguish between wild type and the nonpolar *lpg0178* or Δ*locus* mutants (data not shown), suggesting that the prior transposon insertion led to deleterious polar or non-specific effects on the pathogen. Our experiments may call into question the reported transposon-mediated inactivation of *lpc0263* (ortholog to *lpg0179*) and its negative effect on virulence in the related strain, *L. pneumophila* Corby.[14]

We also investigated whether the BGC had any effect on *in vitro* bacterial growth, survival in water, and biofilm formation relative to wild-type. However, no significant changes were observed under the conditions of our experiments. Finally, representative acyl-histidine **2** was inactive against yeast (*Saccharomyces cerevisiae*, no zone of inhibition around discs loaded with 100 μg of **2**) and bacteria (*E. coli, Staphylococcus aureus*, *Enterococcus faecalis*, and *Pseudomonas aeruginosa,* no zone of inhibition around discs loaded with 50 μg of **2**). Well-known *N*-acyl amides such as anandamide, are known to play key roles in human receptor-mediated cell signaling.[33] Recently Brady and coworkers described an *N*-acyl amide – commendamide – from a human commensal bacteria that activated a NF-κB dependent gene reporter assay in HEK293 cells.[28] Consequently, we screened **2** for NF-κB activation in HEK293 cells (up to 100 μg/ml of **2**), but we did not observe an effect. The pathway's specific role(s) could be functionally redundant with one or more of the many other *Legionella* virulence factors encoded in the genome, and/or the pathway regulates a currently unexamined cellular phenotype(s). These functional contributions remain subjects of future investigations.

Conclusions

In conclusion, we identified a BGC in *L. pneumophila* that produces two distinct groups of aromatic amino acid-derived secondary metabolites, one of which, to the best of our knowledge represents the first *N*-acyl amide metabolites containing L-histidine. Moreover, we observed incorporation of a rare cyclopropane-containing fatty acid that is likely recruited through endogenous phospholipid catabolism. The enzyme responsible for *N*-acylation, *lpg0178* from the ATP-grasp family (PF13535) represents an alternative to the *N*-acyl transferase family (PF13444) responsible for synthesizing *N*-acyl-tyrosines, *N*-acyl-arginines, and commendamide through preactivated carboxylates[28] or the transacylation from glycerophospholipids employed in the biosynthesis of the well-known human *N*-acyl amide anandamide.[34] The biosynthetic genes for the new *N*-acyl-histidine metabolites reported here are up-regulated during intracellular *L. pneumophila* macrophage infection, lending further support to the general notion that structurally diverse *N*-acyl amides could regulate a variety of human cellular signaling programs.

Experimental Section

Bacterial strains and growth conditions.

Legionella pneumophila (subsp. *pneumophila* strain Philadelphia 1) was cultured on N-(2-Acetamido)-2-aminoethanesulfonic acid (ACES)-buffered charcoal yeast extract (CYE) and grown at 37˚C as described.[35] All *L. pneumophila* strains were grown in the presence of 100 μg mL^{-1} streptomycin (Sigma-Aldrich). When necessary, media were supplemented with 5 μg mL^{-1} chloramphenicol (Sigma-Aldrich). *Legionella pneumophila* strain SRS43 was generated by allelic-exchange mutagenesis to incorporate wild-type *thyA* into the Lp02 thymidine auxotroph.[36] Transposon mutants in *lpg0173-lpg0179* were generated by electroporation of pSRS_Cm1, a chloramphenicol resistant derivative of pSAM_Bt[37], into SRS43 and selection of transposon mutants on CYE supplemented with chloramphenicol.

Construction of plasmids for heterologous expression. Genomic DNA from *L. pneumophila* was used as a template for PCR reactions, using the Phusion HF polymerase according to the manufacturer's protocol with primers Leg-A5 and Leg-A3 or LegB5 and LegB3 (Supporting Table 1). The amplification products were purified using a PCR clean-up kit (Macherey-Nagel). The LegA PCR product was digested with NcoI and SacI and ligated into the corresponding sites in pETDuet™-1 (Novagen) to generate pLegA. The LegB PCR product was digested with NdeI and XhoI and ligated into the corresponding sites in pETDuet™-1 to generate pLegB. To generate pLegAB, the LegA PCR product was digested with NcoI and SacI and ligated into the same sites in pLegB. Vector maps are shown in Supporting Figure 1. All constructs were validated by restriction analysis and sequencing and were transformed into chemically competent *E. coli* BAP-1 cells for analysis. The parental plasmid pETDuet™-1 was also transformed into *E. coli* BAP-1 cells to serve as control background for metabolomic analysis.

Construction of deletion mutants for heterologous expression.

1) *pELegAB::Δlpg0174:* The genes *lpg0175-lpg0178* were amplified using the Phusion HF polymerase according to the manufacturer's protocol with primers Leg-Δ174-5 and Leg-A3 (Supporting Table 3). The product was digested with NcoI and SacI and ligated into the same sites in pLegB. 2) *pELegAB::Δlpg0175:* The upstream (*lpg0174*) and downstream (*lpg0176-0178*) regions of *lpg0175* were amplified by PCR using primer pairs Leg-A5/Leg-Δ175-3 and Leg-Δ175-5/Leg-A3. The two products were purified and used as templates in an overlap extension PCR with primers Leg-A5 and Leg-A3. The full-length product was digested with NcoI and SacI and ligated into the corresponding sites in pLegB. 3) *pELegAB::Δlpg0176:* The *lpg0176* gene was deleted from the pLegAB plasmid using a quick-change-like PCR protocol using the primers Leg-Δ176-5 and Leg-Δ176-3. After PCR amplification, the parental plasmid was digested with *Dpn*I. The product was gel purified and transformed into chemically competent *E. coli* DH5α. The deletion was validated by sequencing and the plasmid was transformed into chemically competent *E. coli* BAP-1 for analysis 4) *pELegAB::Δlpg0178:* Genes *lpg0174-lpg0177* were amplified using the Phusion HF polymerase and primers Leg-A5 and Leg-Δ178-3. The product was digested with NcoI and SacI and ligated into the same sites in pLegB. All deletion constructs were validated by sequencing and were transformed into chemically competent *E. coli* BAP-1 for analysis. Vector maps of these constructs are shown in Supporting Figure 4.

Construction of *L. pneumophila* deletion mutants

Clean deletions of *lpg0178* and *lpg0173-lpg0179* were generated by allelic-exchange mutagenesis as previously described.[38] For the *lpg0178* deletion, 5' and 3' flanking regions were amplified using primer pairs 0178KO-5F/0178KO-5R and 0178KO-3F/0178KO-3R, respectively. For the *lpg0173-lpg0179* deletion, 5' and 3' flanking regions were amplified with locusKO-5F/locusKO-5R and locusKO-3F/locusKO-3R, respectively. The digested PCR products were ligated into pSR47s[39] to create deletion constructs pSR47s::Δ*lpg0178* or pSR47s::Δ*locus,* which were maintained in *E. coli* DH5αλ*pir.* Deletion constructs were introduced into wild-type *L. pneumophila* by conjugation. Chromosomal deletions were confirmed by PCR.

***In vitro L. pneumophila* assays**

Replication of *L. pneumophila* wild-type and Δ*locus* in broth culture was performed as previously described.[40] The OD_{600} was measured over 24 hours.

For bacterial survival in water, *L. pneumophila* wild-type and Δ*locus* were resuspended from a 2 day heavy patch in defined water medium (50 mg/L NaCl, 50 mg/L KCl, 20 mg/L KH_2PO_4, pH 6.9). Bacteria were seeded into a 6-well plate and CFUs were enumerated every 7 days for one month as described.[41]

The ability of *L. pneumophila* wild-type or Δ*locus* to form biofilms in the presence of the *Pseudomonas* biofilm inhibitor cis-2-decenoic acid (Sigma) was assayed. Briefly, bacteria were harvested from 2 day heavy patches into AYE media and diluted to OD_{600} = 2.0 Strains were aliquoted into sterile 96-well U-bottom plates and incubated at 25˚C for 6 hours. Supernatant and non-adherent cells were aspirated and AYE supplemented with 0, 1, 10, 100, or 1000 nM *cis*-2-decenoic acid was added to each well (n=8) and plates were incubated at 25˚C for 7 days. Non-adherent cells and supernatants were aspirated and biofilms were assayed as described.[42]

Solvent extraction and identification of pathway-dependent metabolites.

LB agar plates with ampicillin (100 µg/ml) were streaked with glycerol stocks of *E. coli* BAP-1 with pETDuet™-1 or pLegAB. After incubation overnight at 37°C, single colonies were used to inoculate LB liquid cultures (5 ml with ampicillin) in triplicates (or quadruplicates). After overnight growth (37°C, 250 rpm), these samples were used as starter cultures to inoculate fresh LB liquid cultures in triplicates (or quadruplicates). These cultures were grown to approximately OD_{600} = 0.4, at which time they were cooled on ice and induced with IPTG (100 µM). The induced cultures were grown at 30°C and 250 rpm for 2 days. The cultures were vigorously extracted with *n*-BuOH (6 ml added, 4 removed), and the organic fraction was reduced *in vacuo*, re-dissolved in MeOH, and analyzed using a LC-HR-ESI-QTOF-MS (Agilent iFunnel 6550 system) and a single-quadrupole LC-MS (Agilent 6120 system). This protocol was selected as a result of preliminarily screening a number of cultivation conditions: IPTG concentrations (0.1, 0.25, 0.5, and 1.0) or autoinduction growth conditions, temperature (20, 25, 30, and 37°C), base media (M9+casamino acids and LB), and time (1, 2, and 3 days). The pathway-dependent metabolites were identified by high-resolution mass spectrometry using an electrospray ionization (ESI) source on an Agilent iFunnel 6550 quadrupole time-of-flight mass spectrometer (Q-TOF-MS) coupled to an Agilent Infinity 1290 HPLC using a Phenomenex Kinetex C18 column (100 x 2.10 mm, 1.7 µ, 100Å). UPLC conditions consisted of a H_2O:acetonitrile (MeCN) gradient containing 0.1% formic acid: 0-2 min, 5% MeCN; 2-18 min, 5 to 98% MeCN; hold for 5 min, 98% MeCN. Column temperature was set at 25°C and solvent flow was set at 0.3 mL/min. Mass spectra were acquired using Dual Agilent Jet Stream (AJS) ESI in positive mode scanning from 50-1700 *m/z* at 1.00 spectra/second. The capillary and nozzle voltages were set at 3500V and 1000V, respectively. The source parameters were set with a gas temperature at 225°C and flow at 12 L/min, nebulizer at 50 psig, sheath gas temperature at 275°C and flow at 12 L/min. MS data were acquired with MassHunter Workstation Data Acquisition and processed with MassHunter Qualitative Analysis (Agilent Technologies). The centroid MS data was processed with MassHunter Qualitative Analysis and statistically analyzed using MassHunter Mass Profiler Professional (Agilent Technologies). A list of unique ions associated with pLegAB was generated by removing the MOFs found in at least one of the control replicates (pETDuet) and only keeping those that were present in all four experimental biological replicates. This inclusion list was used for subsequent tandem mass spectrometry (MS^2) using targeted auto-MS^2 mode. MS^2 data were acquired in positive mode from 80-1500 *m/z* at a ms/ms scan rate of 1 spectra/sec with fixed collision energies of 10, 20 and 40. The same UPLC method and column conditions were used as described above. The MS^2 data files were used to build mass spectral networking files using the open source software platform Cytoscape version 3.1.0 (http://www.cytoscape.org). Clusters were built based on cosine cutoff of 0.7. Routine screening was performed by monitoring the desired masses on an Agilent 6120 Infinity series quadrupole mass spectrometer coupled to an Agilent 1260 series HPLC system. The mass spectrometer was operated with an atmospheric pressure electrospray ionization (API-ES) source in positive ion mode. LC-MS chromatographic analysis was performed over a Kinetex C18 HPLC column (250 x 4.6 mm, 100Å, 5 µm particle size, Phenomenex) with a H_2O:MeCN gradient at 0.5 mL/min: 0-2 min 5% MeCN; 2-30 min, 5 to 98% MeCN; 30-38 min, hold at 98% MeCN.

Determination of cyclopropane dependency on *cfa*.

To determine whether the cyclopropane group in **1** is dependent on *cfa*, we transformed the Keio parent *E. coli* strain (BW25113)[43] and its *cfa* mutant (JW1653-1)[24] with pLegAB and pTARA:500 (pTara:500 was a gift from Matthew Bennett, Addgene plasmid #60717).[23] Wild type and mutant cultures were analyzed as described under "Solvent extraction and identification of pathway-dependent metabolites" with the exception of a longer post-induction incubation period (one week).

Testing antimicrobial activity.

The antibacterial effect of **2** and **3** was investigated using synthetic materials against *E. coli* BAP1, *Staphylococcus aureus* DSM 20231, *Enterococcus faecalis* DSM 20478, and *Pseudomonas aeruginosa* PAO1 DSM 19880. Glycerol stocks of all four strains were streaked on LB agar plates and grown overnight at 37°C. These cells were used to initiate starter cultures in LB, which were grown overnight at 37°C and 200 rpm The starter cultures were used to inoculate fresh 5 ml LB liquid cultures, and these were grown until approximately OD_{600} = 0.5. Aliquots (200 µl) were then spread uniformly on Mueller Hinton Agar plates. Discs were prepared as blank, 20 µg **3**, 50 µg **3**, 20 µg **2**, and 50 µg **2**. The disc assays were monitored after overnight growth at 37°C.

N-acyl-histidine effects on yeast were investigated using representative **2** (20, 50, and 100 µg loaded on paper discs) against *S. cerevisiae* strain DSY-5 (*MATalpha leu2 trp1 ura3-52 his3::GAL1-GAL4 pep4 prb1-1122)*. Aliquots (100 µl) from an overnight starter culture in YP broth 1:1, 1:10, and 1:100 were spread uniformly onto YP agar plate. After application of the discs, the plates were incubated overnight at 28°C.

Testing NF-κB activation

The reporter cell line used to screen **2** for NF-κB activation (HEK-Blue™ Null2-k; Invivogen, San Diego, California, USA) is a

human embryonic kidney (HEK 293) cell line transfected with an NF-κB/AP-1-SEAP (secreted embryonic alkaline phosphatase) reporter gene. HEK-Blue™ Null2-k was maintained according to the manufacturer's instructions. Two-fold serial dilutions of the positive control, human TNF-α (100 ng/mL in DMSO; Cell Signaling Technology, Danvers, Massachusetts, USA) and of synthetic **2** (100 µg/mL in DMSO) were done in triplicate in a 96-well microplate. HEK-Blue™ Null2-k cells were grown to approximately 80% confluence and the assay was carried out according to the manufacturer's protocol with HEK-Blue™ Detection (Invivogen, San Diego, California, USA), a cell culture medium that allows for colorimetric detection of SEAP. After 16 h of stimulation at 37°C 5% CO_2, SEAP activity was measured with Perkin Elmer EnVision 2100 Plate Reader at 630 nm.

Feeding free fatty acids to heterologous host.
Starter cultures of *E. coli* BAP-1 pLegAB were prepared from single colonies and grown overnight at 37°C and 250 rpm in LB liquid medium supplemented with ampicillin (100 µg/ml). Fresh cultures were inoculated from the starter cultures, and these were grown until OD_{600} = 0.4. All cultures (5 ml each in triplicates) were induced with IPTG (100 µM) and a range of free fatty acids (decanoic acid, octanoic acid, hexanoic acid, *cis*-2-decenoic acid, *trans*-2-decenoic acid, and *cis*-3-decenoic acid) dissolved in dimethyl sulfoxide (DMSO; final supplemented fatty acid concentration, 10 µM). A DMSO solvent control was included as a negative control. The cultures were grown at 30°C for two days before extracting with n-butanol. The extracts were reduced *in vacuo*, re-dissolved in MeOH, and analyzed using a Waters Acquity UPLC instrument coupled to a Bruker Q-TOF maXis Impact mass spectrometer operated in positive electrospray ionization (ESI+) mode. UPLC separation was achieved on an Acquity UPLC HSS T3 column (2.1 mm x 100 mm, 1.8 µm). The column temperature was set at 50°C. Mobile phase A: H2O with 0.1% formic acid; Mobile phase B: MeCN/Methanol 50/50 with 0.1% formic acid. The mobile phase was kept at 0% B for 2 min, followed by a linear gradient to 40% B over 4 min, to 60% over 0.5 min, 88% over 4.5 min, and finally increased to 100% over 0.5 min. The flow rate was 0.4 mL/min and the total analysis time per sample was 21 min. The injection volume was 10 µL, and the sample temperature was maintained at 6°C. Calibration was performed at the beginning of each run by natrium formate acetate (ESI+). A mass range of 50-1000 *m/z* and a sampling rate of 4 Hz were used. The capillary voltage was 4000 V (ESI+). The nebulizing gas pressure was 4 bar and the drying gas flow and temperature were 11 L/min and 220°C, respectively.

Screening for isocyanides using a tetrazine probe.
Starter cultures of *E. coli* BAP-1 with pETDuet, pLegAB, pLegAB-Δ*lpg0176* or the *Vibrio cholerae* isocyanide biosynthetic gene constructs, pEVC1944+pJVC1949[8], were prepared from single colonies and grown overnight at 37°C and 250 rpm in LB liquid medium supplemented with appropriate antibiotics, ampicillin (100 µg/ml) or kanamycin (50 µg/ml). Fresh cultures similarly supplemented with antibiotics were inoculated using the starter cultures and grown until an OD_{600} = 0.4. All cultures (5 ml each in triplicates) were induced with IPTG (100 µM) and grown for two days at 30°C and 250 rpm. Then 3,6-di-2-pyridyl-1,2,4,5 tetrazine (1 mg/ml, 0.5 ml) in MeOH was added and left to react for 1 h at 37°C. The culture was extracted with *n*-butanol (6 ml added, 4 ml removed) and the organic phase was reduced *in vacuo*, re-dissolved in MeOH (500 µl), and analyzed using the method described above in "Solvent extraction and identification of pathway-dependent metabolites."

Isolation of (1) and (2) from *E. coli*.
6 x 1 L of LB liquid media with ampicillin (100 µg/ml) in 4 L Erlenmeyer flasks were inoculated with an overnight culture derived from a single colony. The cultures were grown at 37°C and 250 rpm until OD_{600} = 0.4, at which time they were cooled on ice and induced with IPTG (final conc. 0.1 mM). The cultures were grown for an additional 48 h at 30°C and 250 rpm. The cultures were combined and extracted twice with equal portions of *n*-BuOH (12 L total). The combined organic extracts were filtered, reduced *in vacuo*, re-dissolved in water (500 ml), and fractionated over a C18 column (LiChroprep, RP18, 40-63 µm, Merck). Seven fractions (500 ml each) were collected (H_2O and 10%, 20%, 40%, 60%, 80%, and 100% MeOH, respectively). LC-MS analysis identified the target masses in the 60% and 80% MeOH fractions. These fractions were combined and evaporated *in vacuo* yielding 2 g of crude material. The material was subjected to normal phase LC fractionation on a Biotage system (DCM/MeOH, SNAP-KP-Sil 25g) using a gradient (75 ml/min, 5% MeOH, 30 sec; 5-45%, 4 min, 45-85%, 40 sec; 85%, 1min40sec). Fractions (20ml each) 2-13 contained *m/z* 310 and *m/z* 322 and yielded 600 mg of crude material. The wide retention window indicated acidic functional groups, and further purifications were performed using reversed-phase chromatography. After preparative HPLC (H_2O:MeCN, Agilent Polaris C18-A 5µm 250 x 21.2mm) using a gradient (10 ml/min, 5% MeCN, 2 min; 5-98%, 2-30 min), fractions containing 310 and 322 ions (fractions 24-26, approx. 65% MeCN) were reduced *in vacuo* yielding 43 mg. Semi-preparative RP-HPLC (H_2O:MeCN, Agilent Phenyl-hexyl 5µm 250 x 10 mm) using a gradient (2.5 ml/min, 30% MeCN, 2 min; 30-45%, 2-30 min) gave a mixture of the 310 and 322 ions that then could be separated using RP-HPLC (Phenomenex Luna C8 5µ 250 x 10 mm) and an isocratic (30%) method, yielding **1** (<1 mg) and **2** (2 mg). 1D- and 2D- NMR spectra were recorded on an Agilent 600 MHz NMR equipped with a cold-probe (VnmrJ 3.2 Datastation) in methanol-d_4 . Chemical shifts, coupling constants, and annotation are listed in the supporting material.

Marfey's analysis of (1) and (2).
The isolated metabolites **1** and **2** (approx. 100 µg each) were dissolved in aqueous HCl (6 M) and heated to 115°C for 1 hour and then reduced *in vacuo*. The residue was re-suspended in water (0.5 mL) and lyophilized overnight to remove residual acid. The residue was dissolved in sodium bicarbonate (1 M, 50 µl) and N_α-(2,4-dinitro-5-fluorophenyl)-L-alaninamide (10 mg/ml, 30 µl) in acetone was added. The mixture was heated to 80°C for 2-3 min and then reduced *in vacuo*. The residue was dissolved in H_2O:MeCN (50:50), cleared by centrifugation, and analyzed by

LC-MS. Comparative standards of L-histidine and D-histidine were prepared and analyzed using the same protocols.

Synthesis of cis-3-decenoic acid from 3-decyn-1-ol.

3-decynoic acid: Following the protocol of Qiu et al.[44] a round-bottomed flask (25 ml) was charged with Bobbitt's salt (930 mg, 3.10 mmol, 2.4 eq) and 9:1 mixture of MeCN:H_2O (5 ml). After addition of 3-decyn-1-ol (200 mg, 1.29 mmol) the reaction was left for 48 hours at room temperature. The reaction mixture was concentrated *in vacuo*, diluted with diethylether and extracted three times with aq. NaOH (1 M), then the aqueous phase was acidified with conc. HCl and extracted with diethylether five times. The combined organic phase was washed with brine, dried over Na_2SO_4 and reduced to a slightly yellow solid *in vacuo* (165 mg, 76%). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 3.33 (t, *J* = 2.4 Hz, 2H), 2.20 (tt, *J* = 7.2, 2.5 Hz, 2H), 1.50 (p, *J* = 7.1 Hz, 2H), 1.44 – 1.22 (m, 8H), 0.89 (t, *J* = 6.9 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 175.3, 84.8, 70.6, 31.5, 28.7, 28.7, 26.1, 22.7, 18.9, 14.2. HRMS (ESI): Calc. $C_{10}H_{15}O_2^-$: 167.1078; found: 167.1091 [M - H]$^-$

cis-3-decenoic acid: To a stirring mixture of Nickel(II) acetate tetrahydrate (250 mg) in absolute ethanol (6 ml) saturated with hydrogen was added the filtrate (1.25 ml) of a solution made from $NaBH_4$ (125 mg), aqueous NaOH (2 M, 150 µl) and absolute ethanol (3 ml). To the resulting black suspension was slowly added ethylenediamine (175 µl) and 3-decynoic acid (150 mg). The reaction was left for 1 hour and then diluted with brine (20 ml). After acidifying the mixture with HCl (20%) the product was extracted with diethylether (4 x 50ml). The combined organic phase was washed with brine and dried over Na_2S0_4 yielding cis-3-decenoic acid (124 mg, 82%). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 5.73 – 5.42 (m, 2H), 3.14 (d, *J* = 6.7 Hz, 2H), 2.04 (q, *J* = 7.0 Hz, 2H), 1.45 – 1.17 (m, 8H), 0.88 (t, *J* = 6.7 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.3, 134.4, 120.0, 32.8, 31.8, 29.4, 29.1, 27.6, 22.8, 14.2. HRMS (ESI): Calc. $C_{10}H_{17}O_2^-$: 169.1234; found: 169.1240 [M - H]$^-$

Synthesis of *N*-acyl-L-histidines

The *N*-acyl-L-histidines were prepared using the protocol of Coin et al.[45] and a Fmoc-His(Trt)-Wang resin with the following exceptions: only one coupling reaction was performed with the corresponding fatty acid (decanoic-, octanoic-, or hexanoic acid), no final Fmoc protection for obvious reasons, and final deprotection and cleavage was performed in TFA:H_2O (95:5). The crude cleavage mixture was reduced *in vacuo*, redissolved in aq. AcOH (20%), washed once with DCM and purified by sequential HPLC-purification using a semi-prep LC-MS (Phenomenex, Luna, 5 µ, C18(2), 100 Å, 250 x 10 mm).

C10: ^{1}H NMR (400 MHz, Methanol-d_4) δ 8.41 (d, *J* = 1.4 Hz, 1H), 7.14 (d, *J* = 1.3 Hz, 1H), 4.53 (dd, *J* = 7.7, 5.2 Hz, 1H), 3.19 (dd, *J* = 15.2, 5.0 Hz, 1H), 3.00 (dd, *J* = 15.1, 7.7 Hz, 1H), 2.18 (td, *J* = 7.3, 1.9 Hz, 2H), 1.57 – 1.49 (m, 2H), 1.27 (s, 12H), 0.88 (t, *J* = 7.01, 3H). ^{13}C NMR (101 MHz, MeOD) δ 175.8, 173.5, 134.9, 132.8, 118.2, 54.6, 37.1, 33.1, 30.6, 30.5, 30.5, 30.3, 29.6, 26.9, 23.7, 14.4. HRMS (ESI): Calc. $C_{14}H_{28}N_3O_3^+$: 310.2125; found: 310.2143 [M + H]+.

C8: ^{1}H NMR (400 MHz, Methanol-d_4) δ 8.68 (d, *J* = 1.4 Hz, 1H), 7.25 (d, *J* = 1.3 Hz, 1H), 4.68 (dd, *J* = 8.6, 5.2 Hz, 1H), 3.24 (d*, *J* = 5.2, 1H), 3.04 (dd, *J* = 15.2, 8.7 Hz, 1H), 2.18 (td, *J* = 7.4, 3.9 Hz, 2H), 1.60 – 1.42 (m, 2H), 1.37 – 1.17 (m, 8H), 0.88 (t, *J* = 6.70, 3H). *The second part of an expected dd is obscured by the water peak. ^{13}C NMR (101 MHz, MeOD) δ 176.1, 174.2, 134.9, 132.0, 118.2, 53.2, 36.9, 32.9, 30.2, 28.7, 26.9, 23.7, 14.4. HRMS (ESI): Calc. $C_{12}H_{24}N_3O_3^+$: 282.1812; found: 282.1827 [M + H]+.

C6: ^{1}H NMR (400 MHz, Methanol-d_4) δ 8.52 (s, 1H), 7.19 (s, 1H), 4.58 (dd, *J* = 8.1, 5.2 Hz, 1H), 3.23 (dd, *J* = 15.2, 5.2 Hz, 1H), 3.02 (dd, *J* = 15.1, 8.1 Hz, 1H), 2.17 (td, *J* = 7.4, 1.8 Hz, 2H), 1.53 (p, *J* = 7.6 Hz, 2H), 1.25 (m, 4H), 0.87 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, MeOD) δ 175.9, 175.5, 134.8, 132.5, 118.2, 54.1, 37.0, 32.5, 29.2, 26.5, 23.4, 14.3.
HRMS (ESI): Calc.$C_{12}H_{20}N_3O_3^+$: 254.1499; found: 254.1503 [M + H]+

***Legionella pneumophila* intracellular replication**

NLRC4$^{-/-}$ mice on a C57BL/6 background were generously provided by Richard Flavell and have been described previously.[46] C57BL/6 wild-type mice were purchased from Jackson Laboratories. *NLRC4*$^{-/-}$ BMDMs were generated and maintained as previously described[47].

Differentiated BMDMs were maintained in RPMI (Gibco) supplemented with 10% HI FBS and 7.5% macrophage colony stimulating factor (M-CSF) and seeded at 2 x 10^5/well in 24 well tissue culture treated dishes. Growth curves were performed as previously described and colony forming units (CFU) were enumerated at 1h (t0), 24h, 48h , and 72h p.i.[48]

Acanthamoeba castellanii Neff (ATCC #30010) were maintained in PYG media and cultured at 25˚C as previously described.[49] For infections, cells were seeded into 24 well tissue culture dishes at 2.5 x 10^5/well in PYG medium and allowed to adhere for 2h prior to infection. Cells were washed in warm Ac buffer and infected with the indicated strains at a multiplicity of infection (MOI) of 0.8 in pre-warmed Ac buffer (4 mM $MgSO_4$, 0.4 M $CaCl_2$, 0.1% sodium citrate dehydrate, 0.05 mM $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 2.5 mM NaH_2PO_3, 2.5 mM K_2HPO_3, pH 6.5). At 1h p.i., cells were washed gently 3x with sterile phosphate buffered saline (PBS; Gibco) and Ac buffer was replaced. Bacteria were recovered by collecting supernatants and *A. castellanii* were lysed by passage through a 27 gauge needle three times. Lysates were plated on CYE and CFU were enumerated at 1h (t_0), 24h, 48h, and 72h p.i. Fold replication was calculated by normalizing CFU counts at t_0 to 1. Statistical analyses were performed at each time point using Students *t*-test and GraphPad software.

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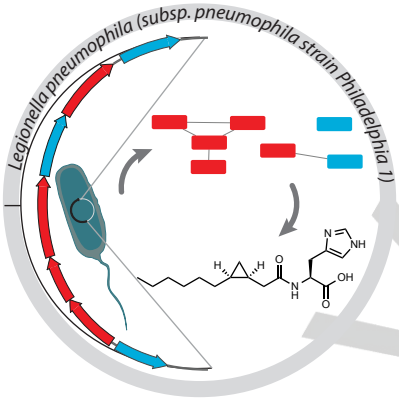
We identified a biosynthetic gene cluster from the human pathogen Legionella pneumophila that produces two distinct groups of secondary metabolites. One of these groups represents a new histidine-derived addition to the biologically important class of N-acyl amides.



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