Legionella shows a diverse secondary metabolism dependent on a broad spectrum Sfp-type phosphopantetheinyl transferase

Nicholas J Tobias 1, Tilman Ahrendt 1, Ursula Schell 2, Melissa Miltenberger 1, Hubert Hilbi 2,3, Helge B Bode Corresponding Author: Helge B Bode
Email address: h.bode@bio.uni-frankfurt.de

Several members of the genus Legionella cause legionnaires’ disease, a potentially debilitating form of pneumonia. Studies frequently focus on the abundant number of virulence factors present in this genus. However, what is often overlooked, is the role of secondary metabolites from Legionella. Following whole genome sequencing, we assembled and annotated the Legionella parisiensis DSM 19216 genome. Together with 14 other members of the Legionella, we performed comparative genomics and analysed the secondary metabolite potential of each strain. We found that Legionella contains a huge variety of biosynthetic gene clusters that are potentially making a significant number of novel natural products with undefined function. Surprisingly, only a single Sfp-like phosphopantetheinyl transferase is found in all Legionella strains analyzed that might be responsible for the activation of all carrier proteins in primary (fatty acid biosynthesis) and secondary metabolism (polyketide and non-ribosomal peptide synthesis). Using conserved active site motifs, we predict some novel compounds that are probably involved in cell-cell communication, differing to known communication systems. We identify several gene clusters, which may represent novel signaling mechanisms and demonstrate the natural product potential of Legionella.
Legionella shows a diverse secondary metabolism dependent on a broad spectrum Sfp-type phosphopantetheinyl transferase

Nicholas J. Tobias¹, Tilman Ahrendt¹, Ursula Schell², Melissa Miltenberger¹, Hubert Hilbi³ & Helge Bode¹,⁴

¹Fachbereich Biowissenschaften, Merck Stiftungsprofessur für Molekulare Biotechnologie, Goethe-Universität Frankfurt, Frankfurt am Main, Germany
²Ludwig-Maximilians University, Max von Pettenkofer Institute, Pettenkoferstr. 9a, 80336 Munich, Germany
³University of Zürich, Institute of Medical Microbiology, Gloriastr. 30, 8006 Zürich, Switzerland
⁴Buchmann Institute for Molecular Life Sciences (BMLS), Goethe-Universität Frankfurt, Frankfurt am Main, Germany

Corresponding author: Helge Bode
Email: h.bode@bio.uni-frankfurt.de
Abstract

Several members of the genus Legionella cause legionnaires’ disease, a potentially debilitating form of pneumonia. Studies frequently focus on the abundant number of virulence factors present in this genus. However, what is often overlooked, is the role of secondary metabolites from Legionella. Following whole genome sequencing, we assembled and annotated the Legionella parisiensis DSM 19216 genome. Together with 14 other members of the Legionella, we performed comparative genomics and analysed the secondary metabolite potential of each strain. We found that Legionella contains a huge variety of biosynthetic gene clusters that are potentially making a significant number of novel natural products with undefined function. Surprisingly, only a single Sfp-like phosphopantetheinyl transferase is found in all Legionella strains analyzed that might be responsible for the activation of all carrier proteins in primary (fatty acid biosynthesis) and secondary metabolism (polyketide and non-ribosomal peptide synthesis). Using conserved active site motifs, we predict some novel compounds that are probably involved in cell-cell communication, differing to known communication systems. We identify several gene clusters, which may represent novel signaling mechanisms and demonstrate the natural product potential of Legionella.
Introduction

The genus of *Legionella* is relatively diverse with 58 member species, 29 of which are known to cause disease in humans (Cunha, Burillo & Bouza, 2016). Legionellosis, infection with a member of the genus, can result in a form of pneumonia known as Legionnaires’ disease or the less severe, flu-like disease known as Pontiac fever. The first *Legionella* was identified following an outbreak of Legionnaires’ disease in 1976, and named *Legionella pneumophila* (Fraser et al., 2010). This species is responsible for a large proportion of Legionnaires’ cases, can often require hospitalization and is particularly dangerous for immuno-compromised patients (Schlossberg & Bonoan, 1998).

All *Legionella* spp. have a common association with water sources, surviving within amoebae, protozoa or slime moulds (Fields, Benson & Besser, 2002). Their association within microbial biofilm communities is also beneficial for their ability to survive and cause disease (Chaabna et al., 2013; Khweek, Dávila & Caution, 2014). This close association between bacteria and protozoan host has led to a number of horizontal gene transfer events, significantly contributing to the intracellular fitness of these bacteria (Chien et al., 2004; Cazalet et al., 2004; Gimenez et al., 2011). Disease outbreaks often occur following contamination of industrial systems that help to spread the bacteria as infectious aerosols (Fraser, 1980; Nguyen et al., 2006). Following phagocytosis by eukaryotic cells, the bacteria are able to survive intracellularly, which is essential for disease progression.

Secondary metabolites are often small chemical compounds produced by a biosynthetic gene cluster (BGC), often consisting of either PKS or NRPS. These compounds are often not essential
for survival but might have significant roles in niche adaptation and virulence. Briefly, PKS and NRPS are multifunctional enzymes that catalyze the condensation of carboxylic acid (PKS) (Hertweck, 2009) or amino acid (NRPS) building blocks (Sieber & Marahiel, 2005). PKS catalyze the formation of C-C bonds via the condensation of malonyl and acyl subunits that are enzyme bound, as in the case of type I PKS, which show similar protein domain architecture to eukaryotic fatty acid synthases. The catalytic functions of PKS and NRPS are organized in modules, with each module responsible for the incorporation and processing of one individual building block (different acyl or malonyl units for PKS or amino acids for NRPS). Due to these similar biochemical principles, hybrids of PKS and NRPS are also possible (Du & Shen, 2001).

The biosynthesis of PKS and NRPS derived natural products as well as fatty acids requires specialized phosphopantetheinyl transferases (PPTases) that catalyze the post-translational transfer of the 4’-phosphopantetheinyl group from coenzyme A (CoA) to acyl (ACP) or peptidyl (PCP) carrier proteins also called thiolation (T) domains. These are components of the enzyme complexes of fatty acid synthases (FAS), polyketide synthases (PKS) and nonribosomal peptide synthetases (NRPS) (Mootz, Finking & Marahiel, 2001; Mofid, Finking & Marahiel, 2002) and covalently link the biosynthesis intermediates to the enzyme complexes. PPTases in bacteria are classified as AcpS (acyl carrier protein synthase) or Sfp (required for surfactin production in *Bacillus subtilis*) enzymes and exhibit different substrate specificities. Sfp-PPTases are monomeric enzymes of approximately 240 aa (Mofid, Finking & Marahiel, 2002) that were shown to activate all kinds of T domains from FAS, PKS and NRPS by attachment of a phosphopantetheinyl group. AcpS PPTases on the other hand, are only half the size and are only functional for ACPs from FAS and type II PKS (Gehring et al., 1997; Mootz, Finking &
Marahiel, 2001; Mofid, Finking & Marahiel, 2002). Therefore, most bacteria (especially those producing secondary metabolites) have two or more PPTases encoded in their genome.

The presence of PKS and NRPS is well established in all types of bacteria, for example, *Streptomyces, Mycobacteria, Myxobacteria, Pseudomonas* and *Bacillus*. Often these products are essential in a particular facet of their respective lifecycles. From *Legionella*, only four BGCs have been explored in detail with three secondary metabolites structurally elucidated to date (Figure 1) (Ahrendt et al., 2013; Shevchuk et al., 2014; Burnside et al., 2015; Johnston et al., 2016a). Legioliulin (1), a product of a trans-AT PKS cluster first identified in *L. dumoffii*, was reported originally in 2004 (Amemura-Maekawa et al., 2004) and biological activity assays failed to determine a role for the compound beyond fluorescence (Ahrendt et al., 2013). This study investigated the possibility that legioliulin is required for intracellular survival and ultimately failed to assign a biological function. On the other hand, a transposon mutagenesis library of *L. pneumophila* revealed a polyketide that interferes with lysosomal degradation during infection of both protozoa and macrophages (Shevchuk et al., 2014). Legiobactin (2) is a siderophore involved in iron sequestration (Cianciotto, 2007) and the unusual polyketide legionellol A (3) is involved in sliding motility and might additionally act as a surfactant (Johnston et al., 2016a). Despite all *Legionella* strains containing several BGCs, no further research has explored the roles of their respective products. To attempt to further explore the possibility that secondary metabolites are an important part of the *Legionella* lifecycle, we performed genome-wide comparisons of 15 genome sequences from *Legionella*, paying particular attention to the prevalence of BGCs. We explore possible structures and functions for these BGCs.
**Materials and Methods**

**Culture conditions and DNA methods**

The *Legionella* strains *L. pneumophila* JR32 (Sadosky, Wiater & Shuman, 1993) and ΔicmT (Segal & Shuman, 1998), *L. longbeachae* NSW150 and *L. parisiensis* DSM 19216 were grown in N-(2-acetamido)-2-aminoethanesulphonic acid (ACES) yeast extract (AYE) broth (Feeley et al., 1979) or on buffered charcoal yeast extract agar (Difco, Detroit, USA) for 3 days at 37°C. *E. coli* BL21 Star (DE3) (Novagen) was cultured in LB medium supplemented with 40 µg/mL kanamycin (Kan) and 100 µg/mL Ampicillin (Amp) (Carl Roth, Karlsruhe, Germany), if necessary. Cells were harvested and DNA was extracted using the Puregene Yeast/Bacteria Kit B (Qiagen) according to the manufacturer’s recommendations.

**Genome sequencing, assembly and annotation**

Shotgun sequencing of *Legionella parisiensis* DSM 19216 was performed using a Genome Sequencer FLX (Roche) by MWG Genomics (Munich). Assembly was performed using the Celera Assembler (v5.3) and quality assessed using QUAST (Gurevich et al., 2013). Sequencing yielded a total of 290,164 reads with average read length of 353 bp. The *L. parisiensis* genome was assembled into a total of 226 contigs (115 ≥ 1kb) with an N50 of 65,672 bp and a predicted genomic coverage of 25. Genome annotation was performed using prokka (v1.12) (Seemann, 2014). Abricate was used to identify common antibiotic resistances (https://github.com/tseemann/abricate).

**Phylogenetic analysis**
Fourteen *Legionella* genomes were downloaded from NCBI (Table 1), their protein fasta files extracted and together with *L. parisiensis*, ortholog families were identified using proteinortho5 (Lechner et al., 2011b). Protein singletons identified in only a single species were removed from further analysis. The presence or absence of all ortholog families were used to generate a gene content tree using the binary function associated with RAxML (Stamatakis, 2014) and the gamma model of rate heterogeneity and a random number seed for parsimony inferences. Protein sequences of the ACPs of *E. coli* (ACPS, WP_000986025.1) and *Bacillus subtilis* (Sfp-like, WP_003234549.1) were taken from the NCBI website and used to identify homologs in each species with Blastp. Protein sequences were aligned using ClustalW, and phylogenetic trees of the PPTases were created using the PhyML plugin attached to Geneious (v6.1.6) (Guindon et al., 2010). Branch formation was supported with bootstrapping (n=1000).

**Cloning and expression of LparPPTase**

pCOLA_Duet1 (Novagen) was used as a vector for overproduction of the PPTase from *L. parisiensis*. The PPTase gene was amplified using primers Lpar_PPtase_Fw_Sacl (GAGCTCGATGATCATTACCGAATTTAACCCT) and Lpar_PPtase_Rv_PstI (GTTCTGAATTAGGGGCAACGTGTCGAC) (synthesized by Sigma-Aldrich, St. Louis, USA).

Both the PCR product obtained and pCOLA_Duet1 were digested with *Sacl* and *PstI* (Fermentas). Digestion products were separated by gel electrophoresis and desired fragments isolated with Gene JETGel extraction kit (Fermentas). Isolated fragments were ligated for 1 h at room temperature using T4-ligase (Fermentas). After ligation, *E. coli* DH10B was transformed with the ligation mixtures in a 1 mm cuvette by electroporation at 1250 V, 200 Ω and 25 μF. Cells were plated on LB-Kan agar and incubated overnight at 37°C. Colonies were picked and
inoculated in LB-Kan media for plasmid extraction. The plasmids obtained were sequenced, and
pCOLA_LparPPTase plasmids transferred into *E. coli* BL21 Star. Positive colonies were picked
and cells were transformed with pUC18_indC (Brachmann et al., 2012) and grown on LB-Kan-
Amp agar. Cells were grown to an OD$_{600}$ of 0.5 at 37°C at which time cultures were induced with
0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Fermentas), and the cultures were
incubated at 16°C overnight. Following induction of LparPPTase in pUC18_indC, cells were
pelleted and resuspended in deionized water for easy visualization of the blue pigment produced
by IndC.

Legionella *in vivo* inhibition.

*L. parisiensis* was grown to an OD$_{600}$ of 0.1 in 200 µl AYE broth in a 96-well plate at 37°C.
Putative PPTase inhibitors 4-6 (Foley et al., 2014) were then added in different concentrations,
and the cells were allowed to grow for 24 hours. For visualization of legioliiulin production in *L.
parisiensis*, the cells were illuminated with long-wave UV-light. The MIC of the PPTase
inhibitors were tested in triplicate on *L. parisiensis*, *L. pneumophila* and *L. longbeachae* using
the OD$_{600}$ value.

Secondary metabolite identification

Secondary metabolites were identified using antiSMASH 3.0 (Weber et al., 2015a) with the
option ClusterFinder algorithm activated. The results from each genome were then aligned using
Mauve (Darling et al., 2004), a BLAST based analysis program, to identify homologous clusters.
Using this method, we assembled some clusters that were split across different contigs by
sequence similarity, additionally taking into account the predicted substrate specificities and
domain modifications from each unassembled module. The sequence for the isocyanide synthase cluster, \textit{isnAB}, was taken from \textit{Xenorhabdus nematophila} (Crawford et al., 2012) and identified in \textit{Legionella} species using BLASTp (v2.2.29) as a part of the BLAST+ suite (Camacho et al., 2009).

Results

Genome of \textit{L. parisiensis}

Purified genomic DNA from \textit{L. parisiensis} DSM 19216 was used for shotgun sequencing. Assembly using Celera (v5.3) revealed a 4,232,283 bp genome with a GC content of 37.98% and was predicted to contain 3,916 protein-coding sequences (CDS). This Whole Genome Shotgun project has been deposited at GenBank under the accession number LSOG00000000. The version described in this paper is version LSOG01000000.

Genome wide analyses

Together with the 14 other \textit{Legionella} genomes (Table 1) we identified all protein ortholog families in \textit{Legionella} (Supplementary Table 1). The core genome of the 15 \textit{Legionella} species consists of 711 coding sequences and includes a type II secretion system as well as the Dot/Icm system. The conserved type II secretion system is essential for intracellular survival and growth (Hales & Shuman, 1999; Polesky et al., 2001; Rossier, Starkenburg & Cianciotto, 2004) as well as promoting growth at low temperatures (Söderberg, Rossier & Cianciotto, 2004). The Dot/Icm system is already known to be ubiquitous in all strains (Feldman et al., 2005). The effectors secreted by this system work in concert to evade the phagosome and form the \textit{Legionella-
containing vacuole allowing the bacteria to grow intracellularly (Isberg, O'Connor & Heidtman, 2009; Ensminger, 2016).

Using the program abricate, we additionally analysed the genomes for possible antibiotic resistance genes. In *L. anisa*, *L. cherrii*, *L. longbeachae*, *L. sainthelensis* and *L. wadsworthii* beta-lactamase resistance was identified with no other antibiotic resistance genes present. However, several multi drug efflux pumps were also found in the genomes (Supplementary Table 2). Using the amino acid sequences of all annotated coding sequences from each strain, we determined ortholog families using proteinortho5 (Lechner et al., 2011a). From these ortholog families, we produced a phylogeny representing the gene content based upon the presence or absence of each protein ortholog family. Following analysis of all *Legionella* strains and their BGCs, we constructed a map of each BGC common to more than a single species based on the protein sequence identity (Figure 2, Figure 3). Bacteriocins are a class of ribosomally synthesized peptides with antibacterial properties. They are classified based on their mode of action and size (Yang et al., 2014) and are typically used to attack other bacteria competing in similar environments (or sometimes have broad-spectrum activity) but contain resistance mechanisms to avoid self-harm (Cotter, Hill & Ross, 2005). This analysis revealed that there is a range of different bacteriocins present in *Legionella* with all species containing at least one cluster with *L. geestiana*, *L. oakridgensis* and *L. shakespearei* containing bacteriocins not present in any other species (Figure 2, Supplementary Figure 1, Supplementary Table 3).

Only a single PPTase was identified in *Legionella*, which activates natural product biosynthesis clusters *in vitro*. 
Interestingly, the ortholog analysis identified only a single Sfp-like phosphopantetheinyl transferase (PPTase) in all of the analyzed *Legionella* genomes (Figure 4). No AcpS-like PPTase that is usually involved in fatty acid biosynthesis exists (Mofid, Finking & Marahiel, 2002). PPTases are required to post-translationally attach a 4’-phosphopantetheine arm from CoA to the serine residue contained in the thiolation (acyl carrier protein (ACP) or peptidyl carrier protein (PCP)) domain and therefore are essential for fatty acid, polyketide and non-ribosomal peptide biosynthesis (Walsh et al., 1997; Stack, Neville & Doyle, 2007).

Unsurprisingly, within this group, *Legionella* PPTases form a distinct branch (Figure 4). To test if the *L. parisiensis* Sfp-like PPTase could activate a NRPS, IndC from *Photorhabdus luminescens* (Brachmann et al., 2012) and the PPTase from *L. parisiensis* were co-produced in *E. coli* BL21 Star. IndC produces the blue pigment indigoidine by condensation of two glutamines. While *indC* is constitutively expressed in this experiment, the *L. parisiensis* PPTase gene expression was under control of an IPTG-inducible promoter. Addition of IPTG and consequent PPTase expression led to blue pigment production (Supplementary Figure 2A). As the *E. coli* Sfp-type PPTase, EntD, is not capable of activating IndC (Brachmann et al., 2012), any production of indigoidine must be activated by the PPTase from *L. parisiensis*. Harvesting and resuspension of the colored cells in water shows a bright blue pigmentation of the IPTG-induced culture (Supplementary Figure 2B).

**Inhibition of legioliulin production and growth in *L. parisiensis***

To rule out the possibility that any PPTase was missed in this analysis, we used 2-pyridinyl-N-(4-aryl)-piperazine-1-carbothioamides (4-6), specific inhibitors of bacterial Sfp-like PPTases (Foley et al., 2014), to shut off legioliulin production. Legioliulin production and growth are
closely linked. Bacterial growth was measured at OD_{600}, and legioliulin production was observed under long-wave UV-light. The addition of 1 µg/mL of inhibitor 4 resulted in a total loss of legioliulin production. We then used different concentrations of 4 to determine substance effectivity (Figure 5). Concentrations as low as 0.4 µg/mL of 4 showed an inhibition in legioliulin biosynthesis and growth. For compounds 5 and 6, initial inhibitory effects were observed at concentrations of 0.75 and 6 µg/mL, respectively. Similarly, growth inhibition was observed for *L. pneumophila* and *L. longbeachae* (Supplementary Table 3).

Biosynthetic gene clusters

During the secondary metabolite analysis, we used antiSMASH to predict BGCs and extracted all those containing predicted siderophore, PKS, NRPS, lantipeptide or bacteriocin clusters. With the optional ClusterFinder algorithm activated (Cimermancic et al., 2014), we also examined all putative and saccharide-like clusters for misclassification (Supplementary Table 3). Strains contained between 15 and 36 BGCs in total with NRPS clusters being the most prevalent. The most widespread PKS, NRPS and siderophore clusters found in *Legionella* are shown in Figure 3 highlighting the overall synteny as well as the domain architecture of the natural product synthases.

Non-ribosomal peptide synthetase product predictions in *Legionella*

PKS and NRPS specificity can often be predicted based upon the DNA sequence and comparisons to experimentally validated studies (Stachelhaus, Mootz & Marahiel, 1999; Challis, Ravel & Townsend, 2000; Yadav, Gokhale & Mohanty, 2003). In the case of the Stachelhaus code, conserved motifs in the adenylation (A) domain are used to predict substrate specificity.
These conserved motifs and their respective specificities were confirmed by single nucleotide mutations resulting in either a loss of, or relaxation of substrate specificity (Stachelhaus, Mootz & Marahiel, 1999). Prediction using a hidden Markov model based approach is also available to predict specificities of either A domains from NRPS or acyltransferase (AT) domains from PKS and is integrated into antiSMASH (Minowa, Araki & Kanehisa, 2007; Weber et al., 2015b). NRSPredictor2, unlike the previous two methods, uses a support vector machine to predict specificities (Röttig et al., 2011). These methods formed the basis to predict the structures of the natural products produced by BGCs in Legionella. In many Legionella BGCs, the specificities for A domains involved in the activation of the correct amino acid in the NRPS could either not be predicted or showed variable results when these different algorithms were used. We therefore only attempted to predict resulting structures where a consensus among the three methods was reached. Several low molecular weight natural products produced from monomodular NRPS could be predicted assuming non-iterative use of these NRPS modules (Figure 6).

Discussion

Secondary metabolism in Legionella is under-pinned by a broad spectrum PPTase

Following the sequencing of the L. parisiensis genome, we noted the presence of 32 BGCs, as predicted by antiSMASH (Supplementary Table 3). We then further investigated a selection of other Legionella strains to obtain a snapshot of the secondary metabolite potential of the genus. Through ortholog clustering we looked specifically for genes that are known to be essential in secondary metabolism. Interestingly, this diversity in secondary metabolites gene clusters appeared to be controlled by a single Sfp-like PPTase in all Legionella strains analyzed, L.Ppt (Legionella PPTase, Figure 4).
This PPTase may therefore be capable of activating all different carrier proteins involved in polyketide and non-ribosomal peptide biosynthesis as well as fatty acid biosynthesis, a part of the primary metabolism as has been seen before (Losick & Isberg, 2006). A precedent for this has been made in *Pseudomonas aeruginosa*, which carries only a single broad spectrum PPTase that is active in both primary and secondary metabolism (Seidle, Couch & Parry, 2006). However, the veracity of this hypothesis is yet to be definitively determined in *Legionella*.

Following identification of only one PPTase, we used an indigoidine production assay to confirm a role for L.Ppt from *L. parisiensis* in secondary metabolism. The enzyme was able to activate the NRPS IndC from *P. luminescens*, even though no NRPS product is known for any *Legionella* strain so far confirming this function. To investigate the effect on suppression of the Sfp-type PPTase, we grew *L. parisiensis* in the presence of Sfp-type PPTase inhibitors (Foley et al., 2014) and showed that legioliulin production, in addition to cell viability, is halted (Supplementary Table 4, Figure 5). The importance of this is that if only a single PPTase controls both primary and secondary metabolism, PPTase inhibitors may be effective as monotherapeutic drugs with multi-target effects (Silver, 2007) resulting from the loss of several functional ACP or PCP proteins, inhibiting essential fatty acid and secondary metabolite biosynthesis. Although fatty acid biosynthesis has been questioned as a general target for antibiotic therapy (Parsons & Rock, 2011), the parallel inhibition of fatty acid, virulence factor and signaling compound biosynthesis might make PPTase inhibitors powerful antibiotics or drugs that could also work against intracellular pathogens, where fatty acid biosynthesis is essential (Yao et al., 2014).

Reconstruction of BGCs and ortholog clustering highlight the diversity of potential secondary metabolites in *Legionella*.
Only the structures of legioluulin (1), legiobactin (2) and legionellol (3) have been solved (Figure 1) while one other PKS derived compound has been implicated in lysosomal degradation (Shevchuk et al., 2014). Legioluulin is a trans-AT PKS derived fluorophore (Figure 3A). However, beyond fluorescence of bacterial strains containing the gene cluster, a biological function was not defined for legioluulin. This is perhaps unsurprising given that only the strains amoebic intracellular growth capabilities were tested while the species has been isolated from both environmental and clinical sources in both fluorescing and non-fluorescing forms (Igel, Helbig & Lück, 2004). The cluster of coding sequences responsible for legionellol, a hydrophilic molecule involved in lipid scaffolding, has been ascribed to a number of small discrete genes (lpq2223-41) coding for different domains in L. pneumophila (Supplementary Figure 3) (Johnston et al., 2016b).

One disadvantage with short read sequencing technologies is that long gene sequences that are prone to containing repetitive sequences may not be properly assembled. This may be the case for the PKS and NRPS gene clusters that we have examined here, as some are known to be highly repetitive such as the mycolactone PKS (Stinear et al., 2004) or the syringopeptin NRPS (Scholz-Schroeder, Soule & Gross, 2007). Although Legionella probably do not contain examples as extreme as mycolactone or syringopeptin, it is possible that the misclassified saccharide-like clusters or some of the contigs containing clusters at their respective termini are in fact collapsed BGCs due to poor assembly. Despite this, we found significant conservation of some BGCs, although this was not always reflected in the phylogenetic tree composed of all coding sequences. For example, Cluster F (Figure 2) is present in species that appear more dissimilar with respect to their gene content. This observation may be in part explained by the
amount of horizontal gene transfer that is reported to occur in this genus leading to a greater
diversity of coding sequences (Gomez-Valero et al., 2011).

Cluster F, the most prevalent cluster, is a NRPS consisting of a single module containing an A, T
and C domain, however it was not limited to a given clade of bacteria suggesting it is probably
either dispensable for growth and survival, or it plays a more general role. Perhaps more
interesting are the clusters that are exclusive to certain clades such as clusters E and K, a NRPS
and type III PKS, respectively as well as clusters M and N, which are both siderophores. The
apparent maintenance of these clusters in specific clades may be representative of essential
functions in their particular environment. However, experimental evidence is needed to verify
the veracity of this hypothesis. Siderophores are a well-known virulence factor of many bacteria
and the structure of legiobactin (2) has already been elucidated in L. pneumophila (Cluster M,
(Burnside et al., 2015)). It is reported as having an identical structure to rhizoferrin (Drechsel et
al., 1991; Burnside et al., 2015) and is essential for ferric iron uptake during infection of the
lungs (Liles, Scheel & Cianciotto, 2000; Robey & Cianciotto, 2002; Allard et al., 2009; Chatfield
et al., 2012).

Several Legionella strains also encode homologs of isnA and isnB that have been shown to be
involved in the biosynthesis of isonitrile containing natural products that are widespread among
bacteria (Brady et al., 2007). Specifically, isnA and isnB encode proteins that, together, produce
an inhibitor of insect phenoloxidase that has been shown to be important in defense against host
immune responses in entomopathogenic bacteria (Brady et al., 2007; Crawford et al., 2012). In
Legionella, a helix-turn-helix domain protein and a cytochrome P450 oxidase are always
associated with the cluster (Figure 3L). In Pseudomonas, the isnAB cluster is part of a larger BGC and does not make the phenoloxidase inhibitor. There, the IsnAB homologs PvcA and PvcB are encoded as a part of the pyoverdine BGC where they are involved in maturation of the siderophore pyoverdine (Drake & Gulick, 2008).

Analysis of clusters B-D reveals the presence of NRPS that are clustered with genes encoding a transcriptional regulator. Although not definitive, this provides evidence supporting a role for these products as novel signaling compounds as seen in other Gram-negative bacteria (Brachmann et al., 2013; Brameyer et al., 2015). If this is indeed the case, its significance lies in the fact that the bacteria occupy a relatively diverse environment and the signals may be specific for their respective niches.

In addition to the more conserved clusters found in several strains, unique clusters have been identified that are present only in individual strains (Supplementary Figure 3). Among them is another trans-AT PKS in L. cherii that might be responsible for the described red fluorescence of this strain that also gave it its name. However, the red fluorescence might also be derived from the legioliulin cluster, also encoded in this genome, when a starting unit other than cinnamic acid is used that could result in a red-shift of the resulting fluorophore. Different PKS/NRPS hybrids are encoded in L. anisa, L. parisiensis and L. longbeachae that additionally encode type I PKSs that could also be involved in the production of unusual fatty acids or lipids required for their particular niche (Supplementary Figure 3).
There are a large number of diverse and interesting BGCs in *Legionella* that have thus far been unexplored. Although few are conserved across species, we cannot rule out the possibility that these BGCs are providing important chemical compounds to their respective strains, whether for signaling, or otherwise. The lack of cluster conservation further reinforces the notion that this genus is a large, untapped reservoir for novel secondary metabolite discovery. Given the association of these bacteria with protozoa in the environment and the interaction of the pathogenic strains with human phagocytic cells, bioactive metabolites originating from this genus may have activity against eukaryotic targets making this an interesting area of future research.

**Availability of supporting data**

Supplementary Table 1 – ortholog families identified in 15 *Legionella* spp.

Supplementary Table 2 – List of coding sequences mentioned in the text and their ortholog in each species

Supplementary Table 3 – Complete list of BGCs

Supplementary Table 4 – Growth inhibition of *L. parisiensis*, *L. pneumophila* and *L. longbeachae* with Sfp-type PPTase inhibitors 4-6.

Supplementary Figure 1 – Cluster organization of clusters O-U

Supplementary Figure 2 – Indigoidine assay

Supplementary Figure 3 – Other clusters unique to some *Legionella*

**List of abbreviations**

PKS – polyketide synthase
Acknowledgements

The authors are grateful to David J. Maloney for samples of PPTase inhibitors 4-6.
References

Ahrendt T, Miltenberger M, Haneburger I, Kirchner F, Kronenwerth M, Brachmann AO, Hilbi H, Bode HB 2013. Biosynthesis of the Natural Fluorophore Legioliulin from Legionella. *Chembiochem: a European journal of chemical biology* 14:1415–1418. DOI: 10.1002/cbic.201300373.

Allard KA, Dao J, Sanjeevaiah P, McCoy-Simandle K, Chatfield CH, Crumrine DS, Castignetti D, Cianciotto NP 2009. Purification of Legiobactin and importance of this siderophore in lung infection by *Legionella pneumophila*. *Infection and immunity* 77:2887–2895. DOI: 10.1128/IAI.00087-09.

Amemura-Maekawa J, Hayakawa Y, Sugie H, Moribayashi A, Kura F, Chang B, Wada A, Watanabe H 2004. Legioliulin, a new isocoumarin compound responsible for blue-white autofluorescence in *Legionella (Floribacter) dumoffii* under long-wavelength UV light. *Biochemical and Biophysical Research Communications* 323:954–959. DOI: 10.1016/j.bbrc.2004.08.180.

Brachmann AO, Brameyer S, Kresovic D, Hitkova I, Kopp Y, Mansce K, Schubert K, Bode HB, Heermann R 2013. Pyrones as bacterial signaling molecules. *Nature chemical biology* 9:573–578. DOI: 10.1038/nchembio.1295.

Brachmann AO, Kirchner F, Kegler C, Kinski SC, Schmitt I, Bode HB 2012. Triggering the production of the cryptic blue pigment indigoidine from *Photorhabdus luminescens*. *Journal of biotechnology* 157:96–99.

Brady, John D Bauer, Michael F Clarke-Pearson A, Daniels R 2007. Natural Products from *isnA*-Containing Biosynthetic Gene Clusters Recovered from the Genomes of Cultured and Uncultured Bacteria. *American Chemical Society*. DOI: 10.1021/ja075492v.

Brameyer S, Kresovic D, Bode HB, Heermann R 2015. Dialkylresorcinols as bacterial signaling molecules. *Proceedings of the National Academy of Sciences of the United States of America* 112:572–577. DOI: 10.1073/pnas.1417685112.

Brenner DJ, Steigerwalt AG, Gorman GW, Wilkinson HW, Bibb WF, Hackel M, Tyndall RL, Campbell J, Feeley JC, Thacker WL, Skaliy P, Martin WT, Brake BJ, Fields BS, Mceachern HV, Corcoran LK 1985. Ten New Species of Legionella. *International journal of systematic and evolutionary microbiology* 35:50–59. DOI: 10.1099/00207713-35-1-50.

Bruner SD, Weber T, Kohli RM, Schwarz D, Marahiel MA, Walsh CT, Stubbs MT 2002. Structural Basis for the Cyclization of the Lipopeptide Antibiotic Surfactin by the Thioesterase Domain SrfTE. *Structure* 10:301–310. DOI: 10.1016/S0969-2126(02)00716-5.

Brzuszkiewicz E, Schulz T, Rydzewski K, Daniel R, Gillmaier N, Dittmann C, Holland G, Schunder E, Lautner M, Eisenreich W, Lück C, Heuner K 2013. *Legionella oakridgensis* ATCC 33761 genome sequence and phenotypic characterization reveals its replication capacity in amoebae. *International Journal of Medical Microbiology* 303:514–528. DOI: 10.1016/j.ijmm.2013.07.003.

Burnside DM, Wu Y, Shafaie S, Cianciotto NP 2015. The *Legionella pneumophila* Siderophore Legiobactin Is a Polycarboxylate That Is Identical in Structure to Rhizoferrin. *Infection and immunity* 83:3937–3945. DOI: 10.1128/IAI.00808-15.

Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, Madden TL 2009. BLAST+: architecture and applications. *BMC Bioinformatics* 10:421. DOI: 10.1186/1471-2105-10-421.

Campbell J, Bibb WF, Lambert MA, Eng S, Steigerwalt AG, Allard J, Moss CW, Brenner DJ
1984. *Legionella sainthelensi*: a new species of *Legionella* isolated from water near Mt. St. Helens. *Applied and environmental microbiology* 47:369–373.

Cazalet C, Gomez-Valero L, Rusniok C, Lomma M, Dervins-Ravault D, Newton HJ, Sansom FM, Jarraud S, Zidane N, Ma L, Bouchier C, Etienne J, Hartland EL, Buchrieser C 2010. Analysis of the *Legionella longbeachae* genome and transcriptome uncovers unique strategies to cause Legionnaires' disease. *PLoS genetics* 6:e1000851. DOI: 10.1371/journal.pgen.1000851.

Cazalet C, Rusniok C, Brüggemann H, Zidane N, Magnier A, Ma L, Tichit M, Jarraud S, Bouchier C, Vandenesch F, Kunst F, Etienne J, Glaser P, Buchrieser C 2004. Evidence in the *Legionella pneumophila* genome for exploitation of host cell functions and high genome plasticity. *Nature Genetics* 36:1165–1173. DOI: 10.1038/ng1447.

Chatfield CH, Mulhern BJ, Viswanathan VK, Cianciotto NP 2012. The major facilitator superfamily-type protein LbtC promotes the utilization of the legiobactin siderophore by *Legionella pneumophila*. *Biometals* 20:323–331. DOI: 10.1007/s10534-006-9057-4.

Chien M, Morozova I, Shi S, Sheng H, Chen J, Gomez SM, Asamani G, Hill K, Nuara J, Feder M, Rineer J, Greenberg JJ, Sheshenko V, Park SH, Zhao B, Teplitskaya E, Edwards JR, Pampou S, Georghiou A, Chou IC, Iannuccilli W, Ulz ME, Kim DH, Geringer-Sameth A, Goldsberry C, Morozov P, Fischer SG, Segal G, Qu X, Rzhetsky A, Zhang P, Cayanis E, De Jong PJ, Ju J, Kalachikov S, Shuman HA, Russo JJ 2004. The Genomic Sequence of the Accidental Pathogen *Legionella pneumophila*. *Science (New York, N.Y.)* 305:1966–1968. DOI: 10.1126/science.1099776.

Cotter PD, Hill C, Ross RP 2005. Bacteriocins: developing innate immunity for food. *Nature reviews. Microbiology* 3:777–788. DOI: 10.1038/nrmicro1273.

Crawford JM, Portmann C, Zhang X, Roetfaers MBJ, Clardy J 2012. Small molecule perimeter defense in entomopathogenic bacteria. *Proceedings of the National Academy of Sciences of the United States of America* 109:10821–10826. DOI: 10.1073/pnas.1201160109.

Cunha BA, Burillo A, Bouza E 2016. Legionnaires' disease. *The Lancet* 387:376–385. DOI: 10.1016/S0140-6736(15)60078-2.

Darling ACE, Mau B, Blattner FR, Perna NT 2004. Mauve: multiple alignment of conserved genomic sequence with rearrangements. *Genome research* 14:1394–1403. DOI: 10.1101/gr.2289704.
New Legionella Species Isolated from Water. *International journal of systematic and evolutionary microbiology* 43:329–337. DOI: 10.1099/00207713-43-2-329.

Drake EJ, Gulick AM 2008. Three-dimensional Structures of *Pseudomonas aeruginosa* PvcA and PvcB, Two Proteins Involved in the Synthesis of 2-Isocyano-6,7-dihydroxycoumarin. *Journal of molecular biology* 384:193–205.

Drechsel H, Metzger J, Freund S, Jung G, Boelaert JR, Winkelmann G 1991. Rhizoferrin — a novel siderophore from the fungus *Rhizopus microsporus* var. *rhizopodiformis*. *Biology of metals* 4:238–243. DOI: 10.1007/BF01141187.

Du L, Shen B 2001. Biosynthesis of hybrid peptide-polyketide natural products. *Current opinion in drug discovery & development* 4:215–228.

Edelstein PH, Brenner DJ, Moss CW, Steigerwalt AG, FRANCIS EM, GEORGE WL 1982. *Legionella wadsworthii* Species Nova: A Cause of Human Pneumonia. *Annals of Internal Medicine* 97:809–813. DOI: 10.7326/0003-4819-97-6-809.

Ensminger AW 2016. *Legionella pneumophila*, armed to the hilt: justifying the largest arsenal of effectors in the bacterial world. *Current Opinion in Microbiology* 29:74–80. DOI: 10.1016/j.mib.2015.11.002.

Feeley JC, Gibson RJ, Gorman GW, Langford NC, Rasheed JK, Mackel DC, Baine WB 1979. Charcoal-yeast extract agar: primary isolation medium for *Legionella pneumophila*. *Journal of clinical microbiology* 10:437–441.

Feldman M, Zusman T, Hagag S, Segal G 2005. Coevolution between nonhomologous but functionally similar proteins and their conserved partners in the *Legionella* pathogenesis system. *Proceedings of the National Academy of Sciences* 102:12206–12211. DOI: 10.1073/pnas.0501850102.

Felnagle EA, Jackson EE, Chan YA, Podevels AM, Berti AD, McMahon MD, Thomas MG 2008. Nonribosomal Peptide Synthetases Involved in the Production of Medically Relevant Natural Products. *Molecular Pharmaceutics* 5:191–211. DOI: 10.1021/mp700137g.

Fields BS, Benson RF, Besser RE 2002. *Legionella* and Legionnaires' disease: 25 years of investigation. *Clinical microbiology reviews* 15:506–526. DOI: 10.1128/CMR.15.3.506-526.2002.

Foley TL, Rai G, Yasar A, Daniel T, Baker HL, Attene-Ramos M, Kosa NM, Leister W, Burkart MD, Jadhav A, Simeonov A, Maloney DJ 2014. 4-(3-Chloro-5-(trifluoromethyl)pyridin-2-yl)-N-(4-methoxypyridin-2-yl)piperazine-1-carbothioamide (ML267), a potent inhibitor of bacterial phosphopantetheinyl transferase that attenuates secondary metabolism and thwarts bacterial growth. *Journal of medicinal chemistry* 57:1063–1078. DOI: 10.1021/jm401752p.

Forseth RR, Amaike S, Schwenk D, Affeltd KJ, Hoffmeister D, Schroeder FC, Keller NP 2013. Homologous NRPS-like Gene Clusters Mediate Redundant Small-Molecule Biosynthesis in *Aspergillus flavus*. *Angewandte Chemie (International ed. in English)* 52:1590–1594. DOI: 10.1002/anie.201207456.

Fraser DW 1980. LEGIONELLOSIS: EVIDENCE OF AIRBORNE TRANSMISSION. *Annals of the New York Academy of Sciences* 353:61–66. DOI: 10.1111/j.1749-6632.1980.tb18906.x.

Fraser DW, Tsai TR, Orenstein W, Parkin WE, Beecham HJ, Sharrar RG, Harris J, Mallison GF, Martin SM, McDade JE, Shepard CC, Brachman PS, Team TFI 2010. Legionnaires' Disease. *dx.doi.org* 297:1189–1197. DOI: 10.1056/NEJM197712012972201.

Gehring AM, Lambalot RH, Vogel KW, Drueckhammer DG, Walsh CT 1997. Ability of
Streptomyces spp. aryl carrier proteins and coenzyme A analogs to serve as substrates in vitro for E. coli holo-ACP synthase. *Chemistry & biology* 4:17–24. DOI: 10.1016/S1074-5521(97)90233-7.

Gimenez G, Bertelli C, Moliner C, Robert C, Raout D, Fournier P-E, Greub G 2011. Insight into cross-talk between intra-amoebal pathogens. *BMC genomics* 12:542. DOI: 10.1186/1471-2164-12-542.

Gomez-Valero L, Rusniok C, Jarraud S, Vacherie B, Rouy Z, Barbe V, Médigue C, Etienne J, Buchrieser C 2011. Extensive recombination events and horizontal gene transfer shaped the *Legionella pneumophila* genomes. *BMC genomics* 12:536. DOI: 10.1186/1471-2164-12-536.

Guindon S, Dufayard J-F, Lefort V, Anisimova M, Hordijk W, Gascuel O 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Systematic biology* 59:307–321. DOI: 10.1093/sysbio/syq010.

Gurevich A, Saveliev V, Vyahhi N, Tesler G 2013. QUAST: quality assessment tool for genome assemblies. *Bioinformatics (Oxford, England)* 29:1072–1075. DOI: 10.1093/bioinformatics/btt086.

Hales LM, Shuman HA 1999. *Legionella pneumophila* contains a type II general secretion pathway required for growth in amoebae as well as for secretion of the Msp protease. *Infection and immunity* 67:3662–3666.

Hertweck C 2009. The Biosynthetic Logic of Polyketide Diversity. *Angewandte Chemie (International ed. in English)* 48:4688–4716. DOI: 10.1002/anie.200806121.

Hoyer KM, Mahlert C, Marahiel MA 2007. The Iterative Gramicidin S Thioesterase Catalyzes Peptide Ligation and Cyclization. *Chemistry & biology* 14:13–22. DOI: 10.1016/j.chembiol.2006.10.011.

Igel L, Helbig JH, Lück PC 2004. Isolation and characterization of a nonfluorescent strain of *Legionella parisiensis*. *Journal of clinical microbiology* 42:2877–2878. DOI: 10.1128/JCM.42.6.2877-2878.2004.

Isberg RR, O’Connor TJ, Heidtman M 2009. The *Legionella pneumophila* replication vacuole: making a cosy niche inside host cells. *Nature reviews. Microbiology* 7:13–24. DOI: 10.1038/nrmicro1967.

Johnston CW, Connaty AD, Skinnider MA, Li Y, Grunwald A, Wyatt MA, Kerr RG, Magarvey NA 2016a. Informatic search strategies to discover analogues and variants of natural product archetypes. *Journal of industrial microbiology & biotechnology* 43:293–298. DOI: 10.1007/s10295-015-1675-9.

Johnston CW, Plumb J, Li X, Grinstein S, Magarvey NA 2016b. Informatic analysis reveals *Legionella* as a source of novel natural products. *Synthetic and Systems Biotechnology*. DOI: 10.1016/j.synbio.2015.12.001.

Khweek AA, Dávila N, Caution K 2014. Biofilm-derived *Legionella pneumophila* evades the innate immune response in macrophages. *Modulation of Host ....* Lechner M, Findeiß S, Steiner L, Marz M, Stadler PF, Prohaska SJ 2011b. Proteinortho : Detection of (Co-)orthologs in large-scale analysis. *BMC Bioinformatics* 12:124. DOI: 10.1186/1471-2105-12-124.

Liles MR, Scheel TA, Cianciotto NP 2000. Discovery of a nonclassical siderophore, legiobactin, produced by strains of *Legionella pneumophila*. *Journal of bacteriology* 182:749–757. DOI: 10.1128/JB.182.3.749-757.2000.

Losick VP, Isberg RR 2006. NF-kappaB translocation prevents host cell death after low-dose challenge by *Legionella pneumophila*. *The Journal of experimental medicine* 203:2177–
Minowa Y, Araki M, Kanehisa M 2007. Comprehensive Analysis of Distinctive Polyketide and Nonribosomal Peptide Structural Motifs Encoded in Microbial Genomes. *Journal of molecular biology* 368:1500–1517. DOI: 10.1016/j.jmb.2007.02.099.

Mofid MR, Finking R, Marahiel MA 2002. Recognition of hybrid peptidyl carrier proteins/acyl carrier proteins in nonribosomal peptide synthetase modules by the 4′-phosphopantetheinyl transferases AcpS and Sfp. *Journal of Biological Chemistry* 277:17023–17031. DOI: 10.1074/jbc.M200120200.

Mootz HD, Finking R, Marahiel MA 2001. 4′-phosphopantetheine transfer in primary and secondary metabolism of *Bacillus subtilis*. *Journal of Biological Chemistry* 276:37289–37298. DOI: 10.1074/jbc.M103556200.

Nguyen TMN, Ilef D, Rouil L, Campese C, Che D, Haeghebaert S, Ganiayre F, Marcel F, Etienne J, Desenclos J-C 2006. A community-wide outbreak of legionnaires disease linked to industrial cooling towers--how far can contaminated aerosols spread? *Journal of Infectious Diseases* 193:102–111. DOI: 10.1086/498575.

Pagnier I, Croce O, Robert C, Raoult D 2014. Genome sequence of *Legionella anisa*, isolated from a respiratory sample, using an amoeba coculture procedure. *Genome* ... 14:544–549.

Parsons JB, Rock CO 2011. Is bacterial fatty acid synthesis a valid target for antibacterial drug discovery? *Current Opinion in Microbiology* 14:544–549.

Polesky AH, Ross JT, Falkow S, Tompkins LS 2001. Identification of *Legionella pneumophila* genes important for infection of amoebas by signature-tagged mutagenesis. *Infection and immunity* 69:977–987. DOI: 10.1128/IAI.69.2.977-987.2001.

Rizzardi K, Winiecka-Krusnell J, Ramliden M, Alm E, Andersson S, Byfors S 2015. *Legionella norrlandica* sp. nov., isolated from the biopurification systems of wood processing plants. *International journal of systematic and evolutionary microbiology* 65:598–603. DOI: 10.1099/ijs.0.068940-0.

Robey M, Cianciotto NP 2002. *Legionella pneumophila* feoAB promotes ferrous iron uptake and intracellular infection. *Infection and immunity* 70:5659–5669. DOI: 10.1128/IAI.70.10.5659-5669.2002.

Rossier O, Starkenburg SR, Cianciotto NP 2004. *Legionella pneumophila* type II protein secretion promotes virulence in the A/J mouse model of Legionnaires' disease pneumonia. *Infection and immunity* 72:310–321. DOI: 10.1128/IAI.72.1.310-321.2004.

Röttig M, Medema MH, Blin K, Weber T, Rausch C, Kohlbacher O 2011. NRPSpredictor2—a web server for predicting NRPS adenylation domain specificity. *Nucleic acids research* 39:gkr323–W367. DOI: 10.1093/nar/gkr323.

Sadosky AB, Wiater LA, Shuman HA 1993. Identification of *Legionella pneumophila* genes required for growth within and killing of human macrophages. *Infection and immunity* 61:5361–5373.

Schlossberg D, Bonoan J 1998. *Legionella* and immunosuppression. *Seminars in respiratory infections* 13:128–131.

Scholz-Schroeder BK, Soule JD, Gross DC 2007. The sypA, sypB, and sypC Synthetase Genes Encode Twenty-Two Modules Involved in the Nonribosomal Peptide Synthesis of Syringopeptin by *Pseudomonas syringae* pv. syringae B301D. *dx.doi.org* 16:271–280. DOI: 10.1094/MPMI.2003.16.4.271.

Seemann T 2014. Prokka: rapid prokaryotic genome annotation. *Bioinformatics (Oxford, England)* 30:2068–2069. DOI: 10.1093/bioinformatics/btu153.
Segal G, Shuman HA 1998. Intracellular multiplication and human macrophage killing by *Legionella pneumophila* are inhibited by conjugal components of IncQ plasmid RSF1010. *Molecular microbiology* 30:197–208.

Seidle HF, Couch RD, Parry RJ 2006. Characterization of a nonspecific phosphopantetheinyl transferase from *Pseudomonas syringae* pv. *syringae* FF5. *Archives of biochemistry and biophysics* 446:167–174.

Shaw-Reid CA, Kelleher NL, Losey HC, Gehring AM, Berg C, Walsh CT 1999. Assembly line enzymology by multimodular nonribosomal peptide synthetases: the thioesterase domain of *E. coli* EntF catalyzes both elongation and cyclolactonization. *Chemistry & biology* 6:385–400. DOI: 10.1016/S1074-5521(99)80050-7.

Shevchuk O, Pägelow D, Rasch J, Döhrmann S, Günther G, Hoppe J, Ünal CM, Bronietzki M, Gutierrez MG, Steinert M 2014. Polyketide synthase (PKS) reduces fusion of *Legionella pneumophila*-containing vacuoles with lysosomes and contributes to bacterial competitiveness during infection. *International Journal of Medical Microbiology* 304:1169–1181. DOI: 10.1016/j.ijmm.2014.08.010.

Sieber SA, Marahiel MA 2005. Molecular Mechanisms Underlying Nonribosomal Peptide Synthesis: Approaches to New Antibiotics. *Chemical Reviews* 105:715–738. DOI: 10.1021/cr0301191.

Silver LL 2007. Multi-targeting by monotherapeutic antibacterials. *Nature Reviews Drug Discovery* 6:41–55. DOI: 10.1038/nrd2202.

Söderberg MA, Rossier O, Cianciotto NP 2004. The type II protein secretion system of *Legionella pneumophila* promotes growth at low temperatures. *Journal of bacteriology* 186:3712–3720. DOI: 10.1128/JB.186.12.3712-3720.2004.

Stachelhaus T, Mootz HD, Marahiel MA 1999. The specificity-conferring code of adenylation domains in nonribosomal peptide synthetases. *Chemistry & biology* 6:493–505. DOI: 10.1016/S1074-5521(99)80082-9.

Stack D, Neville C, Doyle S 2007. Nonribosomal peptide synthesis in *Aspergillus fumigatus* and other fungi. *Microbiology* 153:1297–1306. DOI: 10.1099/mic.0.2006/006908-0.

Stamatakis A 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics (Oxford, England)* 30:1312–1313. DOI: 10.1093/bioinformatics/btu033.

Stinear TP, Mve-Obiang A, Small PLC, Frigui W, Pryor MJ, Brosch R, Jenkin GA, Johnson PDR, Davies JK, Lee RE, Adusumilli S, Garnier T, Haydock SF, Leadlay PF, Cole ST 2004. Giant plasmid-encoded polyketide synthases produce the macrolide toxin of *Mycobacterium ulcerans*. *Proceedings of the National Academy of Sciences* 101:1345–1349. DOI: 10.1073/pnas.0305877101.

Thacker WL, Benson RF, Hawes L, Gidding H, Dwyer B, Mayberry WR, Brenner DJ 1991. *Legionella fairfieldensis* sp. nov. isolated from cooling tower waters in Australia. *Journal of clinical microbiology* 29:475–478.

Thacker WL, Dyke JW, Benson RF, Havlichek DH, Robinson-Dunn B, Stiefele H, Schneider W, Moss CW, Mayberry WR, Brenner DJ 1992. *Legionella lansingensis* sp. nov. isolated from a patient with pneumonia and underlying chronic lymphocytic leukemia. *Journal of clinical microbiology* 30:2398–2401.

Verma UK, Brenner DJ, Thacker WL, Benson RF, Vesey G, Kurtz JB, Dennis PJL, Steigerwalt AG, Robinson JS, Moss CW 1992. *Legionella shakespearei* sp. nov., Isolated From Cooling Tower Water. *International journal of systematic and evolutionary microbiology* 42:404–
Post-translational modification of polyketide and nonribosomal peptide synthases. Current opinion in chemical biology 1:309–315. DOI: 10.1016/S1367-5931(97)80067-1.

Weber T, Blin K, Duddela S, Krug D, Kim HU, Bruccoleri R, Lee SY, Fischbach MA, Müller R, Wohlleben W, Breitling R, Takano E, Medema MH 2015b. antiSMASH 3.0-a comprehensive resource for the genome mining of biosynthetic gene clusters. Nucleic acids research 43:W237–43. DOI: 10.1093/nar/gkv437.

Wilkinson HW, Drasar V, Thacker WL, Benson RF, Schindler J, Potuznikova B, Mayberry WR, Brenner DJ 1988. Legionella moravica sp. Nov. and Legionella brunensis sp. Nov. Isolated from cooling-tower water. Annales de l'Institut Pasteur / Microbiologie 139:393–402. DOI: 10.1016/0769-2609(88)90102-0.

Yadav G, Gokhale RS, Mohanty D 2003. Computational Approach for Prediction of Domain Organization and Substrate Specificity of Modular Polyketide Synthases. Journal of molecular biology 328:335–363.

Yang S-C, Lin C-H, Sung CT, Fang J-Y 2014. Antibacterial activities of bacteriocins: application in foods and pharmaceuticals. Frontiers in Microbiology 5:12. DOI: 10.3389/fmicb.2014.00241.

Yao J, Abdelrahman YM, Robertson RM, Cox JV, Belland RJ, White SW, Rock CO 2014. Type II fatty acid synthesis is essential for the replication of Chlamydia trachomatis. The Journal of biological chemistry 289:22365–22376. DOI: 10.1074/jbc.M114.584185.
Figure 1. Structures of the known *Legionella* natural products legioluvin, legionellol and legibactin as well as PPTase inhibitor used in this study (4-6).
Figure 2. *Legionella* phylogeny based on presence or absence of ortholog families together with a summary of orthologous BGCs found in two or more *Legionella* species. BGCs were identified using antiSMASH (Weber et al., 2015b) and nucleotide sequences were aligned using Mauve (Darling et al., 2004) to determine those that were similar. Ortholog presence was first determined using proteinortho5 (Lechner et al., 2011a). The gene content tree was then constructed using RAxML, based on the presence or absence of each ortholog. BGCs are separated according to the class of compound produced. Cluster letters refer to those genetic schematics shown in Figure 3 and compound numbers refer to those found in Figures 1 & 6. A full list of BGCs can be found in Supplementary Table 3. The gene cluster encoding IsnAB is not detected by antiSMASH but is a known BGC responsible for the biosynthesis of isonitrile containing compounds that are widespread in bacteria (Brady et al., 2007).
Figure 3. Representative examples of BGCs found in multiple *Legionella* species as identified in Figure 2. Protein domain architecture as determined from NCBI’s conserved domain database for NRPS (green) and PKS (red) encoding genes are also shown. Each circle represents an individual domain of the respective PKS or NRPS (domains not to scale). The PKS from *L. pneumophila* contains a C-terminal condensation domain typical of those seen in NRPSs, which is also capable of polyketide chain release. Clusters O-U can be found in Supplementary Figure 1. All clusters are in Supplementary Table 3.
Figure 4. Maximum likelihood phylogeny created using PhyML of PPTases identified in *Legionella* genomes and their relationship to a selection of PPTases from other bacteria. Scale represents amino acid substitutions per amino acid position. Bootstrapping (n=1000) was used to support branch formation.
Figure 5. Inhibition of legioliolulin production resulting in fluorescence (at 366 nm) in *L.*
parisiensis by PPTase inhibitors 4-6.
Figure 6. Theoretical structures of compounds 7-10 predicted from the clusters B-D, G and I (monomodular NRPS), shown in Figure 2. Monomodular NRPS are predicted to produce modified amino acids or dipeptide derivatives that have also been identified in different fungi (Forseth et al., 2013). In a relatively rarely described phenomenon, NRPS domains may be re-used during product biosynthesis resulting in peptides longer than expected from the NRPS domain architecture. An example of such an iterative use is due to the action of the thioesterase domain which, following a single round of biosynthesis, must oligomerize the enzyme bound peptide product before release from the NRPS (Shaw-Reid et al., 1999; Bruner et al., 2002; Hoyer, Mahlert & Marahiel, 2007; Felnagle et al., 2008). Due to the relative infrequency that this happens, we assumed non-iterative use of domains for all structural predictions. Cluster B (Figure 3) encodes a NRPS/PKS hybrid that is suggested to produce a valine elongated by a single polyketide elongation using malonyl-CoA with the resulting product, dependent on the thioesterase (TE) function, might be linear (7a) or cyclic (7b). Cluster C encodes a monomodular NRPS that is predicted to produce a N-formylated amino acid that is either reduced by the C-terminal reduction (Red) domain to the aldehyde (8a), or the alcohol (8b) that can then by cyclized non-enzymatically to form an oxazoline ring (8c). The acylated amino acid derived from cluster D can undergo similar transformation resulting in structurally related compounds (9a, 9b, 9c). Cluster G and I are very similar to C but the NRPS is terminated by a TE domain resulting again in either a linear (10a) or cyclic product (10b).
Table 1. All genome details for *Legionella* spp. used in this study.

| Species                              | Genome Accession No. | Source                      | Reference                        |
|--------------------------------------|----------------------|-----------------------------|----------------------------------|
| *Legionella anisa* str. Linanisette  | NZ_CANP00000000.1    | Clinical sample             | (Pagnier et al., 2014)           |
| *Legionella cherrii* DSM19213        | NZ_JHYM00000000.1    | Thermally altered water     | (Brenner et al., 1985)           |
| *Legionella drancourtii* LLAP12      | NZ_ACUL00000000.2    | Environmental water source  | (Gimenez et al., 2011)           |
| *Legionella faeifieldsensis* ATCC49588 | NZ_JHYC00000000.1   | Cooling tower               | (Thacker et al., 1991)           |
| *Legionella geestiana* LLAP12        | NZ_JHYN00000000.1    | Domestic hot water          | (Dennis et al., 1993)            |
| *Legionella lansingensis* DSM19556   | NZ_JHWF00000000.1    | Clinical sample             | (Thacker et al., 1992)           |
| *Legionella longbechiae* NSW150      | NC_013861.1, NC_014544.1 | Clinical sample          | (Cazalet et al., 2010)           |
| *Legionella moravica* DSM19234       | NZ_AUHS00000000.1    | Cooling tower               | (Wilkinson et al., 1988)         |
| *Legionella norrlandica* strain LEGN | NZ_JNCF00000000.1    | Biopurification system of wood processing plant | (Rizzardi et al., 2015) |
| *Legionella oakridgensis* ATCC33761  | NZ_CP004006.1, NZ_CP004007.1 | Cooling tower               | (Brzuszkiewicz et al., 2013)     |
| *Legionella pneumophila* subsp.      | NC_002942.5          | Clinical sample             | (Chien et al., 2004)             |
| *pneumophila* str. Philadelphia 1    |                      |                             |                                  |
| *Legionella saintheleensis* ATCC35248 | NZ_JHXP00000000.1   | Surface water               | (Campbell et al., 1984)          |
| *Legionella shakespearei* DSM23087   | NZ_AREN00000000.1    | Cooling tower               | (Verma et al., 1992)             |
| *Legionella wadsworthii* DSM21896    | NZ_JNIA00000000.1    | Clinical isolate            | (Edelstein et al., 1982)         |
| *Legionella parisiensis* DSM19216    | LSOG00000000         | Cooling tower               | This study                       |