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Hypothesis

Presence of Bacterial Virulence Gene Homologues in the dibenzo-p-dioxins degrading bacterium *Sphingomonas wittichii*

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Abstract:
Sphingomonas wittichii, a close relative of the human pathogen *Sphingomonas paucimobilis,* is a microorganism of great interest to the bioremediation community for its ability of biodegradation to a large number of toxic polychlorinated dioxins. In the present study we investigated the presence of different virulence factors and genes in *S. wittichii.* We utilized phylogenetic, comparative genomics and bioinformatics analysis to investigate the potentiality of *S. wittichii* as a potential virulent pathogen. The 16SrDNA phylogenetic tree showed that the closest bacterial taxon to *S. wittichii* is *Brucella* followed by *Helicobacter*, *Campylobacter*, *Pseudomonas* then *Legionella.* Despite their close phylogenetic relationship, *S. wittichii* did not share any virulence factors with *Helicobacter* or *Campylobacter.* On the contrary, in spite of the phylogenetic divergence between *S. wittichii* and *Pseudomonas spp.*, they shared many major virulence factors, such as, adherence, antiphagocytosis, Iron uptake, proteases and quorum sensing. *S. wittichii* contains several major virulence factors resembling *Pseudomonas sp*., *Legionella sp*., *Brucella sp*. and *Bordetella sp*. virulence factors. Similarity of virulence factors did not match phylogenetic relationships. These findings suggest horizontal gene transfer of virulence factors rather than sharing a common pathogenic ancestor. *S. wittichii* is a potential virulent bacterium. Another possibility is that reductive evolution process attenuated *S. wittichii* pathogenic capabilities. Thus plenty of care must be taken when using this bacterium in soil remediation purposes.

Keywords: *Sphingomonas wittichii*, Virulence factors, Phylogenetics, Comparative genomics, Bioinformatics, *Pseudomonas sp*.

Abbreviations:
GDLs: Glycosphingolipids; 16S rDNA: 16S ribosomal RNA; NJ: Neighbor-Joining; MCL: Maximum Composite Likelihood; taxid: Taxon identity; cAMP: Cyclic adenosine phosphate; HSF: Histamine sensitizing factor; LPF: Lymphocytosis promoting factor (LPF); IAP: Islet-activating protein.

Background:
Sphingomonas wittichii is a bacterium of immense importance in the context of bioremediation because of its ability to biodegrade large number of toxic polychlorinated dioxins and to utilize both non-chlorinated dibenzo-p-dioxin and non-chlorinated dibenzofuran as a growth substrate and a sole source of carbon and energy **[1].** *S. wittichii* belongs to the genus *Sphingomonas* that is a group of Gram-negative, rod-shaped, non-sporeforming, chemoheterotrophic, strictly aerobic bacterium that produces yellow or off white pigmented colonies. The distinctive features of *Sphingomonas* include its possession of ubiquinone 10 as its major respiratory quinone, the presence of glycosphingolipids (GDLs) in their cell envelopes and its metabolic versatility **[2].**

Members of the *Sphingomonas* genus are gaining popularity for their significant ability to degrade numerous recalcitrant compounds **[3–6]**. The completed 5,915,246-bp genome of *S. wittichii* consists of a main chromosome of 5,382,261 bp and two mega-plasmids, designated as pSWIT01 and pSWIT02 with sizes of 310,228 bp and 222,757 bp respectively **[7].** *S. wittichii* is also known to be a compelling degrader of toxic dioxin pollutants, as it fully degrades the organic backbone of the dibenzo-p-dioxin structure **[6, 8].** This bacterium was largely isolated for its ability

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to grow on dioxin-like compounds as the sole carbon and energy source **[6]**. And since its isolation, compelling research has shown that *S. wittichii* can biodegrade a larger number and greater diversity of chlorinated diaryl ethers than any other known bacterium **[6, 9, 10].**

In a previous study, we confirmed the presence of many virulence factors in *S. paucimobilis,* a human pathogen that had been frequently isolated from Diabetic foot ulcer patients **[11]** and is also closely related to *S. wittichii.* Henceforth, in this study, we aimed to investigate the pathogenic potentials of *Sphingomonas wittichii* by employing comparative genomics and bioinformatics techniques. This is the first study to investigate the presence of bacterial virulence factors in the presumed environmentally friendly bacterium, *S. wittichii.*

Methodology:

Phylogenetic relationships reconstruction:

We acquired partial 16S rDNA sequences of selected Sphingomonas species and pathogenic bacteria from the GenBank (**Supporting information Table 3**). These sequences were then aligned using the Bioedit built-in clustal W program (gap opening penalty = 10, gap extension penalty = 5, delay divergent sequences = 40%). We compared the resulting alignments and the final alignments were improved manually and prepared in FASTA, MEGA formats using format converter tool v2.2.5 available online at: http://www.hiv.lanl.gov/content/sequence/FORMAT_CONV E RSION/form.html.

In order to establish the phylogenetic relationships among taxa, tree was constructed using the Maximum Likelihood (ML) method based on the Tamura-Nei model and Jukes Cantor the best fit to the data according to AIC criterion **[12].** The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then the topology was selected with superior log likelihood value. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 18 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. MEGA6 (program/software/tool) was used to conduct evolutionary analyses and pairwise distance **[13].**

Comparative genomics analysis:

Virulence genes sequences and functions, corresponding to different major bacterial virulence factors of chosen pathogens, were acquired as described before by Saeb *et al.* **[11].** Sequences were acquired from GenBank then compared and validated with virulence factors of pathogenic bacteria data base **Supporting information Table** 2 that shows the tested major pathogenic virulence factors. Selected gene sequences were tested against available *Sphingomonas* gene information using *Sphingomonas* Nucleotide BLAST tool. The search set all *Sphingomonas* complete genomes and selected organism was *Sphingomonas* (taxid: 13687). Because of evolutionary divergence of the tested and query taxa we used BLASTN that is optimized for fairly similar sequences.

Table 1: Major pathogenic taxa used in the comparative analysis against *Sphingomonas wittichii*

Genus	Species	Host	Disease
Brucella	*B. abortus*	Human and Cattle	Brucellosis, Osteoarthritis, endocarditis and several neurological disorders
	B. canis	Human and Dogs	Brucellosis, Osteoarthritis, endocarditis and several neurological disorders
	B. melitensis	Human Goats and Sheep	Brucellosis, Osteoarthritis, endocarditis and several neurological disorders
	B. ovis	Sheep	Brucellosis, Osteoarthritis, endocarditis and several neurological disorders
	B. suis	Human and Pigs	Brucellosis, Osteoarthritis, endocarditis and several neurological disorders
Helicobacter	*H. acinonychis*	Humans and other mammals	Bacterial carcinogen, Gastroduodenal diseases
	H. hepaticus	Humans and other mammals	Bacterial carcinogen, Gastroduodenal diseases
	H. pylori	Humans and other mammals	Bacterial carcinogen, Gastroduodenal diseases
Campylobacter	*C. fetus*	Humans	Bacterial gastroenteritis
	C. jejuni	Humans	Guillain-Barre syndrome (GBS)
Legionella	*L. pneumophila*	Humans and protozoa	Legionnaires' Disease
Pseudomonas	*P. aeruginosa*	Human	Eye, burn and wound infections
	P. syringae	Plant	Bacterial speck and bacterial blight

Table 2: Suggested *Sphingomonas wittichii* toxin information in relation to *Bordetella pertussis* toxins.

Bordetella Toxin	Related Gene	Product name	Genbank ID	*Shingomonas* spp		
				E value	Identity (%)	Accession
Cya (Invasive	cyaA	bifunctional hemolysin-adenylate cyclase precursor	33591934	2.00E-12	73	NC_009511.1
Adenylate cyclase/	cyaD	cyclolysin secretion protein	33591936	8.00E-04	81	NC_009511.1
haemolysin)	cyaE	cyclolysin secretion protein	33591937	2.00E-04	89	NC_009511.1
	ptlC	putative bacterial secretion system protein	33594645	3.00E-06	88	NC_009511.1
Ptx (Pertussis toxin)	ptlF	putative bacterial secretion system protein	33594649	5.00E-04	76	NC_009511.1
	ptlH	putative bacterial secretion system protein	33594651	3.00E-08	75	NC_009511.1

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Table 3: Comparative analysis *Sphingomonas wittichii* against major bacterial virulence factors and functions

Bacterial Taxa	Major virulence factors (VFs)	Sub VFs	Related Gene	E value	Identity (%)	Accession #
Brucella	Intra-cellular survival and Immuno-modulatory activity	Mannose-1-phosphate guanylyl-transferase	*manC*	3.0E-09	77	NC_009511.1
		Phospho-glucomutase	*pgm*	0	74	NC_009511.1
	Secretion system	VirB type IV	*BMEII0026*	6.0E-04	90	NC_020561.1
		VirB type IV	*BMEII0035*	4.0E-06	72	NC_020561.1
Legionella	Adherence	Hsp60	*htpB*	6.0E-19	64	NC_009511.1
	Motility	Flagella	*fliP*	1.0E-4	66	NC_009511.1
	Stress protein	SodB	*sodB*	3.0E-04	76	NC_009511.1
Pseudomonas	Adherence	Flagella	*fleQ*	4.0E-65	72	NC_009511.1
			flgE	5.0E-19	67	NC_009511.1
			flgF	7.0E-14	71	NC_009511.1
			flgG	3.0E-44	68	NC_009511.1
			flgH	3.0E-25	72	NC_009511.1
			flgI	6.0E-87	68	NC_009511.1
			flgK	1.0E-04	94	NC_009511.1
			flhA	2.0E-139	70	NC_009511.1
			flhB	2.0E-11	70	NC_009511.1
			flhF	6.0E-05	89	NC_020561.1
			fliC	1.0E-32	72	NC_009511.1
			fliE	6.0E-04	74	NC_020561.1
			fliF	3.0E-04	66	NC_020561.1
			fliG	8.0E-9	63	NC_009511.1
			fliI	1.0E-115	71	NC_009511.1
			fliN	3.0E-16	70	NC_009511.1
			fliP	7.0E-84	72	NC_009511.1
			fliQ	2.0E-08	84	NC_020561.1
			fliR	7.0E-08	72	NC_020561.1
	Adherence	LPS (lipopolysaccharide)	*waaG*	4.0E-07	76	NC_020561.1
			waaP	1.0E-04	83	NC_020561.1
		Type IV pili	*chpA*	2.0E-13	69	NC_009511.1
			chpC	8.0E-05	82	NC_020561.1
			fimU	3.0E-04	93	NC_009511.1
			pilB	3.0E-04	93	NC_009511.1
			pilG	2.0E-04	79	NC_009511.1
			pilH	2.0E-04	77	NC_009511.1
			pilJ	2.0E-19	76	NC_009511.1
			pilK	3.0E-06	83	NC_020561.1
			pilQ	1.0E-09	73	NC_009511.1
			pilR	9.0E-54	70	NC_009511.1
			pilS	2.0E-05	74	NC_009511.1
			pilT	1.0E-07	83	NC_009511.1
			pilU	1.0E-12	75	NC_009511.1
			pilV	3.0E-04	78	NC_020561.1
			pilW	1.0E-05	88	NC_009511.1
	Antiphagocytosis	Alginate	*Alg44*	7.0E-04	85	NC_020561.1
			algA	1.0E-21	65	NC_009511.1
			algB	2.0E-36	69	NC_009511.1
			algI	1.0E-59	67	NC_009511.1
			algJ	7.0E-04	94	NC_009511.1
			algP	1.0E-07	80	CP006644.1
	Biosurfactant	Rhamnolipid	*rhlA*	5.0E-04	89	NC_009511.1
	Iron uptake	Pyochelin	*fptA*	6.0E-08	86	NC_009511.1
			pchA	8.0E-04	93	NC_009511.1
			pchB	6.0E-04	67	NC_020561.1
			pchC	1.0E-04	80	NC_009511.1
			pchD	1.0E-09	67	NC_009511.1
			pchE	6.0E-05	81	NC_009511.1
			pchF	1.0E-07	85	NC_009511.1
			pchG	1.0E-05	79	NC_009511.1
			pchH	4.0E-09	67	NC_009511.1
			pchI	6.0E-13	74	NC_009511.1
		Pyoverdine	*fpvA*	3.0E-12	68	NC_020561.1
			pvdA	8.0E-04	86	NC_009511.1
			pvdD	1.0E-04	91	NC_020561.1
			pvdE	7.0E-06	82	NC_009511.1
	Pigment	Pyocyanin	*phzM*	5.0E-05	77	NC_009511.1
		Pyocyanin	*phzS*	6.0E-05	68	NC_009511.1
	Protease	Alkaline protease	*aprA*	5.0E-07	76	NC_009511.1
		LasA	*lasA*	7.0E-04	86	NC_009511.1
	Regulation	Quorum sensing	*rhlL*	3.0E-04	93	NC_009511.1
	Secretion system	xcp secretion system	*rhlR*	5.0E-09	72	NC_009511.1
			xcpQ	2.0E-46	70	NC_009511.1
			xcpR	3.0E-174	72	NC_009511.1
			xcpS	1.0E-44	66	NC_009511.1
			xcpT	3.0E-23	69	NC_009511.1
			xcpU	2.0E-07	78	NC_020561.1
			xcpW	4.0E-04	73	NC_009511.1
			xcpX	6.0E-04	88	NC_009511.1

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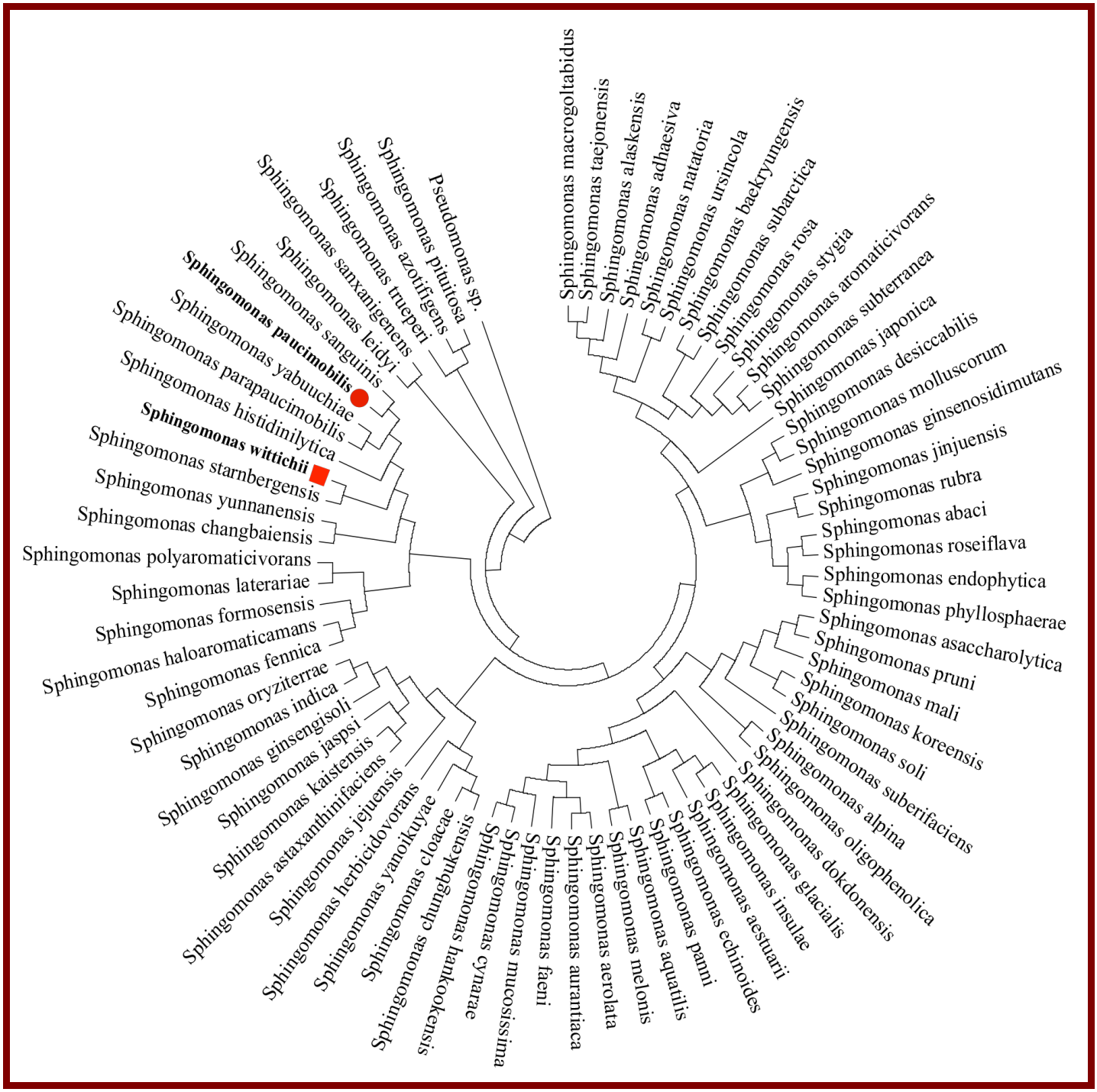

Figure 1: Partial 16SrDNA based phylogenetic tree for 74 species of genus *Sphingomonas* including the human pathogen *S. paucimobilis*.

Results and Discussion:

In this study, the presence of major known bacterial virulence factors in *Sphingomonas wittichii* was investigated. Phylogenetic relationships among 74 species of genus *Sphingomonas,* including the human pathogen *Sphingomonas paucimobilis* were reconstructed. **Figure 1** shows the Partial 16SrDNA based Maximum Likelihood phylogenetic tree for all 74 species of the genus *Sphingomonas*. This figure also shows that *Sphingomonas wittichii* is a relatively close taxon to *S. starnbergensis, S. histidinilytica, S. parapaucimobilis, S. yabuuchiae* and the human pathogen *S. paucimobilis.* The pair wise genetic distance analysis was performed which showed the overall value of distance analysis among *Sphingomonas* species to be 0.05. The individual values of pairwise distance between of *S. wittichii* with *S.*

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sanguinis, S. histidinilytica, S. parapaucimobilis, S. paucimobilis, S. yabuuchiae and the distantly related species *S. mali* were 0.016, 0.026, 0.048, 0.042, 0.042 and 0.06 **(Supporting information Table 1)** respectively. The *S. starnbergensis* is a novel type of freshwater bacterium isolated from the prealpine mesotrophic Starnberger See (Bavaria, southern Germany). This species showed 95.3 % sequence similarity with *S. paucimobilis* DSM 1098(T), the type species of the genus Sphingomonas **[14]**. While, the *S. histidinilytica* was isolated from an open hexachlorocyclohexane (HCH) dump site at Ummari village in Lucknow, India. It showed 16S rDNA similarity of 99.4 %, with Sphingomonas wittichii DSM 6014(T) **[15].** Moreover, *S. yabuuchiae* was identified from samples taken from the Russian space laboratory Mir. As shown in our results (Figure 1), *S. yabuuchiae* 16S rDNA sequence formed a coherent cluster with *Sphingomonas sanguinis, Sphingomonas parapaucimobilis, Sphingomonas paucimobilis* and Sphingomonas roseiflava with sequence similarity of 97·5–98·6% **[16].** *Sphingomonas wittichii* also came in a coherent cluster with *S. parapaucimobilis* and *S. paucimobilis*, the only two species that are considered of human clinical significance **[17].**

The Maximum Likelihood method was used to construct a phylogenetic tree using partial 16S rDNA sequences of selected pathogenic bacteria as mentioned above in (**Figure 2**) and this was specifically done in order to make a guided decision for the choice of pathogenic bacteria species that will be used in the following comparative genomics analysis with *Sphingomonas wittichii*. This phylogenetic tree showed that *Brucella sp.* was the closest bacterial taxon to *Sphingomonas,* followed by *Helicobacter spp., Campylobacter sp., Pseudomonas sp.,* and then *Legionella sp.* Based on these suggested phylogenetic relationships, the following bacterial species, *Brucella sp., Helicobacter sp., Campylobacter sp., Pseudomonas sp.* and *Legionella sp*, were selected for further comparative genomic and bioinformatics analysis.

Table 1 shows the selected bacterial genera with its corresponding species, hosts, and the diseases. All the chosen pathogens were mainly human, animal, protozoa, and plants pathogens. **(Table 2)** shows the virulent factors acquired by the chosen pathogens that were tested for its presence in *S. wittichii* genomic information. The major categories of bacterial virulence factors include adherence, endotoxin production, adherence, mobility, secretion systems and quorum sensing.

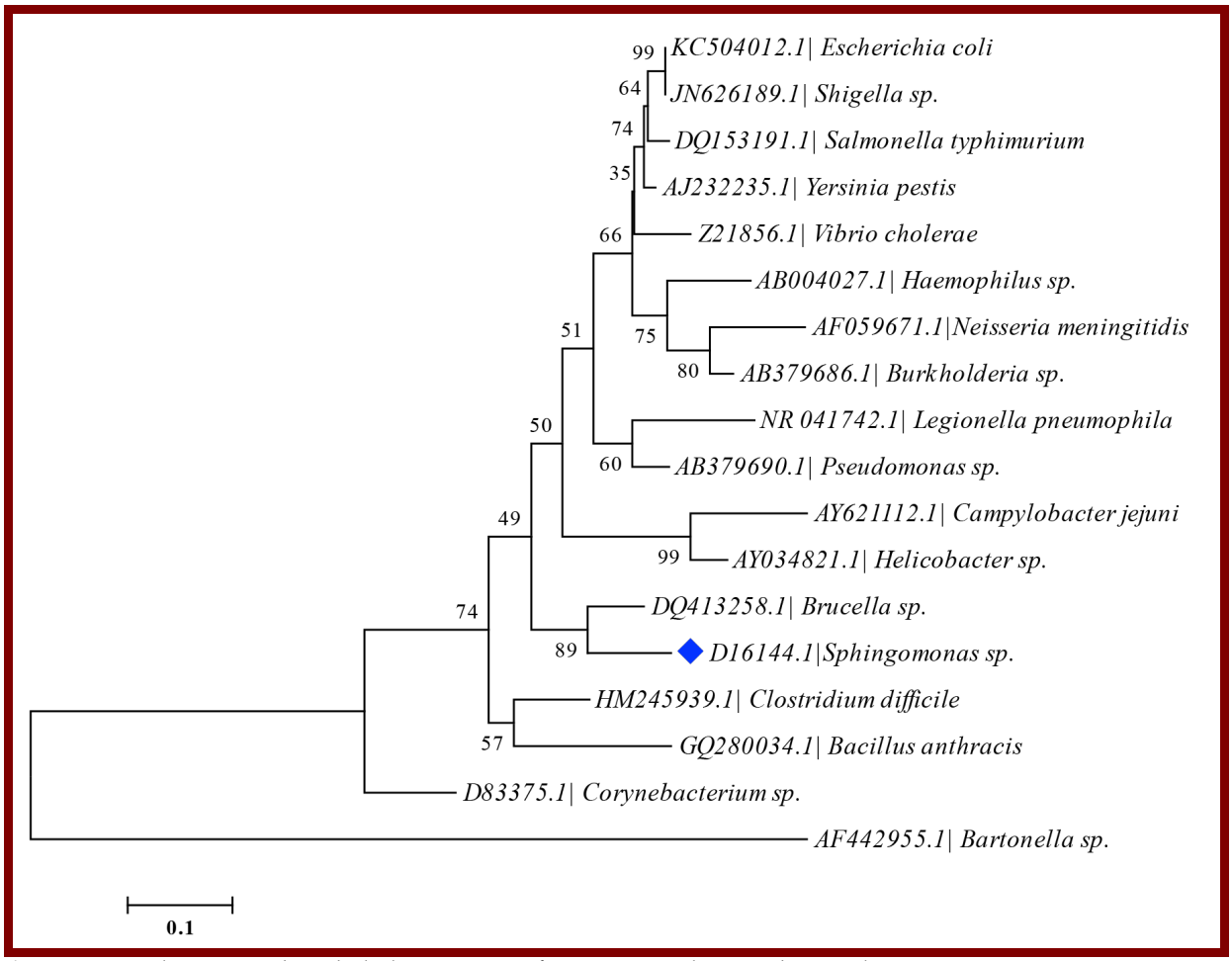

Figure 2: Partial 16SrDNA based phylogenetic tree for a major pathogenic bacterial taxa.

Table 3 and Figure 3 presents the shared virulence factors among *S. wittichii* and the selected five bacterial pathogens. Results in **Table 3** showed that *S. wittichii* shares the genes accountable for intracellular survival capability (manC and pgm) with *Brucella* sp. with e-values ranging from 0 to 3.00E-09 **[11–13].** In addition, *Sphingomonas spp.*, shares the genes encoding for Type IV secretion system such as BMEII0026 with *Brucella sp.* with e-value of 6.00E-04 and identity similarity of 90%. On the contrary, *S. wittichii* does not share any virulence factors with *Helicobacter spp.* or *Campylobacter sp.* despite their close phylogenetic relationship. Moreover, *S. wittichii* shared *Legionella sp.* genes accountable for adherence and motility, namely, htpB and flip. In addition, *S. wittichii* and *Legionella sp.* shared the gene responsible for stress tolerance and sodB. The sodB encodes for superoxide dismutase which is a cytoplasmic iron superoxide dismutase important for intracellular survival and transmission **[18].**

Notwithstanding of the phylogenetic divergence between *S. wittichii* and *Pseudomonas sp.*, it was noticed that they share several major virulence factors such as, adherence, antiphagocytosis, iron uptake, proteases, quorum sensing. *S. wittichii* and *Pseudomonas sp.* shared 19 genes of flagella formation (adherence) including flgK with e-value of 1.00E-04 and identity similarity of 94% and flgF with e-value of 6.00E-05 and identity similarity of 89%. The Flagella formation plays an important role as a virulence factor that enable motility toward the infection site, biofilm formation and several other pathogenic adaptations **[19–23].** Moreover, *S. wittichii* and *Pseudomonas sp.* shared many genes implicated in type IV pili biogenesis and mechanical function of pili, such as fimU with e-value of 3.00E-04 and pilB with e-value of 3.00E-04 and 93% identity similarity. The type IV pili system plays an important role in adherence by assisting the pathogens to attach with their host cells and the twitching motility that allows the bacteria to move along the cell surface and in biofilm formation **[20, 24–28].** Moreover, they shared two genes waaG and waaP implicated in lipopolysaccharide production that also play role in adherence ability. Additionally, *S. wittichii* and *Pseudomonas sp.* share many genes implicated in antiphagocytosis through alginate production. They shared six alginate genes including algJ with e-value of 7.00E-04 and identity similarity of 94% and alg44 with e-value of 7.00E-04 and identity similarity of 85%. Alginate production allows pathogens to form bacterial biofilms and contributes to the persistence of bacteria in the lung by acting as an adhesin, which prevents the bacteria from being expelled from the infection site. The alginate slime layer makes it more difficult for phagocytes to ingest and kill the bacteria **[29–33].**

Another important bacterial virulence factor shared between *S. wittichii* and *Pseudomonas sp.* is quorum-sensing ability. *S. wittichii* has both rhlL and rhlR with e-values of 3.00E-4 and 5.00E-9, respectively. Thus *S. wittichii* possess only rhl system of quorum sensing. Whilst in *Pseudomonas sp.* quorum sensing consists of two separate but interrelated systems, namely; las and rhl which are found to regulate the production of multiple virulence factors and are also crucial for proper biofilm formation **[34–36].** In addition, *S. wittichii* and *Pseudomonas sp.* share seven genes encoding for xcp secretion system (Type II secretion system) including xcpX and xcpR with e-values of 1.00E-5 and 3.00E-174, respectively. The xcp secretion system is found to be responsible for secretion of toxins and enzymes into the extracellular fluid **[37, 38].** It was also observed that both *S. wittichii* and *Pseudomonas sp.* share several genes involved in Iron uptake using both Pyochelin (10 genes) and Pyoverdine (4 genes). The Pyochelin is effective at enhancing iron uptake in *P. aeruginosa*, catalyzes the formation of tissue-damaging free radicals and also binds other transition metals (e.g. Mo (IV), Co (II)) with appreciable affinity and is also implicated in the delivery of both Co (II) and Mo (IV) to *P. aeruginosa* cells **[39, 40].** The Pyoverdine is effective at acquiring iron from Transferrin and Lactoferrin. Moreover, Pyoverdine is cytotoxic due to its ability to stimulate the production of reactive oxygen species **[41, 42].**

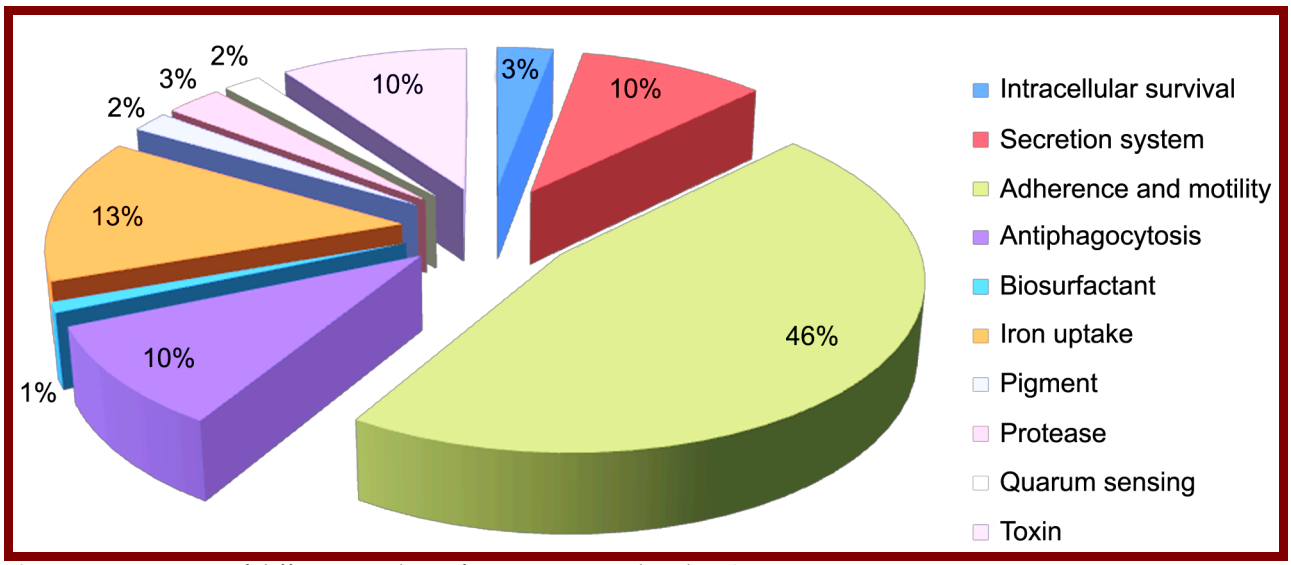

Figure 3: Percentage of different virulence factors associated with *Sphingomonas spp.*

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Since production of bacterial toxins is very important aspect of virulence, we extended our comparative analysis in order to investigate the presence of toxin related genes in *S. wittichii*. **Table 2** shows toxins that were found to share between *S. wittichii* and *Bordetella pertussis*, the strictly aerobic Gram-negative coccobacilli pathogen. *B. pertussis* is a strict human pathogen causing whooping cough, a highly contagious respiratory disease marked by severe, spasmodic coughing episodes **[43–45].** It was also observed that *S. wittichii* contains genes for Invasive Adenylate cyclase /haemolysin, cyclolysin secretion protein which is a bi-functional toxin harboring both adenylate cyclase and hemolytic activities and functions primarily as an anti-inflammatory factor **[46–48].** Moreover, *Sphingomonas spp.* contains genes responsible for Pertussis toxin and its secretion system which assists in the attachment of *B. pertussis* to ciliated respiratory cells, important immunogen and activate cyclic Adenosine Phosphate (cAMP), Histamine Sensitising Factor (HSF), Lymphocytosis Promoting Factor (LPF), Islet-activating protein (IAP), interferes with leucocyte function and is haemolytic **[49–53].**

Results of this study showed that *S. wittichii* contains several major virulence factors mainly resembling *Pseudomonas sp.* Other virulence factors from *Legionella sp., Brucella sp.* and *Bordetella sp.*, have also been observed. Moreover, the similarity of virulence factors did not correspond to the phylogenetic relationships. These findings suggest horizontal gene transfer of virulence factors rather than sharing a common pathogenic ancestor. The other possible scenario is that *S. wittichii* went through a reductive evolution process that attenuated its pathogenic capabilities. In both cases we suggest that lots of care must be taken when releasing *S. wittichii* higher concentrations in the process environmental remediation.

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