bioRxiv preprint doi: https://doi.org/10.1101/028977; this version posted October 13, 2015. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

CRISPR system acquisition and evolution of an obligate intracellular *Chlamydia*-related bacterium

Claire Bertelli[1,2], Ousmane Cissé[1], Brigida Rusconi[1], Carole Kebbi-Beghdadi[1], Antony Croxatto[1], Alexander Goesmann[3], François Collyn[1], Gilbert Greub[°1]

[1] Center for Research on Intracellular Bacteria, Institute of Microbiology, University Hospital Center and University of Lausanne, Lausanne, Switzerland, [2] SIB Swiss Institute of Bioinformatics, Lausanne, Switzerland [3] Bioinformatics and Systems Biology, Justus-Liebig-University Giessen, Gießen, Germany

[°]Corresponding author:

Gilbert Greub, MD PhD

Institute of Microbiology

University of Lausanne

1011 Lausanne

SWITZERLAND

Phone : +41-21-314 49 79

Fax : +41-21-341 40 60

e-mail : gilbert.greub@chuv.ch

Running Title: The Protochlamydia CRISPR system

bioRxiv preprint doi: https://doi.org/10.1101/028977; this version posted October 13, 2015. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

ABSTRACT

Recently, a new *Chlamydia*-related organism, *Protochlamydia naegleriophila* KNic, was discovered within a *Naegleria* amoeba. To decipher the mechanisms at play in the modeling of genomes from the *Protochlamydia* genus, we sequenced *de novo* the full genome of *Pr. naegleriophila* combining the advantages of two second-generation sequencing technologies. The assembled complete genome comprises a 2,885,111 bp chromosome and a 145,285 bp megaplasmid. For the first time within the *Chlamydiales* order, a CRISPR system, the immune system of bacteria, was discovered on the chromosome. It is composed of a small CRISPR locus comprising eight repeats and the associated *cas* and *cse* genes of the subtype I-E. A CRISPR locus was also found within *Chlamydia* sp. Diamant, another *Pr. naegleriophila* strain whose genome was recently released, suggesting that the CRISPR system was acquired by a common ancestor of these two members of *Pr. naegleriophila*, after the divergence from *Pr. amoebophila.* The plasmid encodes an F-type conjugative system similar to that found in the Pam100G genomic island of *Pr. amoebophila* suggesting an acquisition of this conjugative system before the divergence of both *Protochlamydia* species and the integration of a putative *Pr. amoebophila* plasmid into its main chromosome giving rise to the Pam100G genomic island. Overall, this new *Pr. naegleriophila* genome sequence enables to investigate further the dynamic processes shaping the genomes of *Chlamydia*-related bacteria.

bioRxiv preprint doi: https://doi.org/10.1101/028977; this version posted October 13, 2015. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

INTRODUCTION

A large diversity prevails in the order *Chlamydiales,* as suggested by the discovery of a large number of *Chlamydia* and *Chlamydia*-related bacteria belonging to nine different families (Greub 2010; Everett et al. 1999; Horn 2011) and the cross-examination of metagenomics data (Lagkouvardos et al. 2014). The family *Parachlamydiaceae* comprises five genera that are each represented by a small number of isolated strains. The genus *Protochlamydia* was lately enriched by the isolation of a *Naegleria* endosymbiont that presented 97.6% identity in the 16S rRNA with *Pr. amoebophila* UWE25 and was thus named *Pr. naegleriophila* strain KNic (Casson et al. 2008). Since other members of the *Parachlamydiaceae* family were suspected to be associated with lung infections (Greub 2009), a diagnostic PCR specific for *Pr. naegleriophila* was developed and applied to bronchoalveolar lavages. *Pr. naegleriophila* DNA was detected in the bronchoalveolar lavage of an immunocompromised patient with pneumonia by two PCRs targeting different genomic regions and the presence of the bacterium in the sample was confirmed by direct immunofluorescence (Casson et al. 2008). These results indicated a potential role of *Pr. naegleriophila* in lower respiratory tract infections.

A recent study including *Chlamydia* genomes and other members of the *Planctomycetes-Verrucomicrobia-Chlamydia* superphylum suggested that the branch leading to *Chlamydia* was shaped mainly by genome reduction and evidenced limited occurrence of gene birth, duplication and transfer (Kamneva et al. 2012), as it is the case in other strict intracellular pathogens (Darby et al. 2007). On the contrary, the occurrence of large families of paralogs in the genome of *Chlamydia*-related bacteria suggested an evolution by extensive gene duplication (Domman et al. 2014; Eugster et al. 2007). The chromosome sequence of *Pr. amoebophila* UWE25 exhibited little evidence for the occurrence of lateral gene transfer (Horn et al. 2004). However, a number of probable lateral gene transfers were identified between *Parachlamydia* and other amoeba-infecting bacteria such as *Legionella* (Gimenez et al. 2011), a process that may take place within the amoeba itself (Bertelli & Greub 2012). The *Pr. amoebophila* genome presents a genomic island (Pam100G) that encodes a type IV secretion system of the F-type that might be involved in conjugative DNA transfer (Greub et al. 2004). A similar system was found on the plasmid of *Simkania negevensis* (Collingro et al. 2011) and a partial operon was described in *Parachlamydia acanthamoebae* (Greub et al. 2009), suggesting active DNA transfer capabilities in the ancestor of the *Chlamydiales* and some of its descendants.

Small interspaced repetitions were initially observed in *E. coli* (Ishino et al. 1987) and they were then named CRISPR, an acronym for Clustered Regularly Interspaced Short Palindromic Repeats (Jansen et al. 2002). Although found in 50% of bacteria and in 90% of archaea (Weinberger et al. 2012), a CRISPR system had never been previously reported in a member of the order *Chlamydiales*

bioRxiv preprint doi: https://doi.org/10.1101/028977; this version posted October 13, 2015. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

(Makarova et al. 2011). The CRISPR locus usually consists of a variable number (up to 587) of 23-47bp repeats with some dyad symmetry, but not truly palindromic, interspaced by 21-72 bp spacers (Horvath & Barrangou 2010). Associated with these repeats are 2 core cas genes and additional subtype-specific genes putatively providing mechanistic specificity (Koonin & Makarova 2013). Similarity between spacers and extrachromosomal elements first suggested a role in immunity against phage infection and more generally against conjugation or transformation by acquisition of external DNA (Bolotin et al. 2005). The CRISPR-Cas system was shown to mediate an antiviral response thus inducing resistance to phage infection (Deveau et al. 2010), notably in *E. coli* (Brouns et al. 2008). More recently, CRISPR-Cas systems were shown to regulate stress-related response, changing gene expression and virulence traits in several pathogens among which is the intracellular bacteria *Francisella novicida* (Sampson & Weiss 2014; Louwen et al. 2014).

In this contribution, we sequenced and analyzed the complete genome of *Pr. naegleriophila* strain KNic and discovered two potentially antagonistic systems, a type IV secretion system likely implicated in conjugative DNA transfer and a CRISPR system that generally controls foreign DNA acquisition. Furthermore, the complete genome sequence of a new species within the genus *Protochlamydia* offered the possibility to look into the genome dynamics throughout evolution by comparing *Pr. naegleriophila* KNic gene content and genome architecture to its closest relatives of the family *Parachlamydiaceae*.

RESULTS

Chromosome features and evolution

Pr. naegleriophila KNic possesses a 2’885’090 bp circular chromosome with a mean GC content of 42.7%. The genome size and the GC content are surprisingly high compared to the most closely-related species, *Pr. amoebophila* (**Table 1**), but it is consistent with its closest relative *Chlamydia sp.* Diamant, another *Pr. naegleriophila* strain (hereafter referred to as *Pr. naegleriophila* Diamant). The chromosome of *Pr. naegleriophila* strain KNic is predicted to encode 2,415 proteins and exhibits four ribosomal operons and 43 tRNAs, more than any other *Chlamydiales* (**Table 1**). Two types of spacers are found between the 16S and the 23S rRNA: either a simple intergenic spacer or a spacer containing two tRNAs for Ala and Ile.

The cumulative G versus C nucleotide bias (GC skew) presents a typical pyramidal shape (**Figure S1**) that is expected in the absence of particular large genomic islands and confirms the assembly accuracy. The GC skew of *Pr. naegleriophila* is smoother than that of *Pr. amoebophila*, and does not

bioRxiv preprint doi: https://doi.org/10.1101/028977; this version posted October 13, 2015. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

present the small inversion in the slope that is caused by the *Pr. amoebophila* genomic island (**Figure S1**) (Greub et al. 2004). The replication origin (*ori*) and the terminus of replication (*ter*), at the minimum and maximum of the curve (**Figure S1**), respectively, show an almost perfectly balanced chromosome with 49.8% of the base on one arm, i.e., between *ori* and *ter*, and 50.2% on the other arm, i.e., between *ter* and *ori*.

The two strains of *Pr. naegleriophila* are highly collinear as shown in the alignment of available complete and nearly complete (<5 contigs) genomes of the family *Parachlamydiaceae* (**Figure 1**). Within genus comparison of *Pr. naegleriophila* and *Pr. amoebophila* shows the occurrence of 13 recombination and inversion events. As expected, further distantly-related organisms from a different genus exhibit a lower collinearity and an increasing number of recombination events, which is correlated to the phylogenetic distance.

pPNK is an F-type conjugative megaplasmid

The bacterial chromosome was circularized, leaving behind several contigs with a 23-fold coverage, slightly higher than the 16-fold chromosomal coverage. These contigs formed a 145,285 bp large plasmid - the largest known plasmid in the order *Chlamydiales*. The plasmid pPNK presents a GC content of 37.2% and includes 160 genes among which are several transposase and integrase remnants, doc proteins, and systems for the maintenance of the plasmid (*parA* and PNK_p0119) that are all characteristic of extra-chromosomal elements.

The plasmid also encodes a type IV secretion system with highest similarity to the F-type system found in the genomic island of *Pr. amoebophila* UWE25 (Greub et al. 2004), in the plasmid of *Chlamydia* sp. Rubis (hereafter named *Parachlamydia sp.* Rubis) and *S. negevensis* (Collingro et al. 2011) and to the remnants *traU, traN* and *traF* present in members of the family *Parachlamydiaceae* (Greub et al. 2009; Collingro et al. 2011) (**Figure 2**). The type IV secretion system of *Parachlamydia* sp. Rubis is located on a 30 kb long contig that should be circularized as a small plasmid to retain the colinearity of the *tra* operon with other bacteria. This small plasmid would therefore contain almost exclusively the *tra* operon as well as core genes for plasmid replication such as *parA*. *Parachlamydia* sp. Rubis and KNic *tra* operons share a striking colinearity. The comparison of gene conservation shows that *traN* has undergone different rearrangements in both *Pr. amoebophila* strains, and *traC* was split in strain R18. On the other hand, *Parachlamydia sp.* Rubis, *S.* negevensis and *Pr. naegleriophila* KNic, the three bacteria that possess the *tra* operon on a plasmid, retained intact genes. Moreover, these bacteria present Ti-type *traA* and *traD* genes downstream that share similarity to and other amoeba-infecting bacteria such as *Rickettsia bellii* and *Legionella* spp..

bioRxiv preprint doi: https://doi.org/10.1101/028977; this version posted October 13, 2015. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

138 **A CRISPR –Cas system for the first time within *Chlamydiales***

139 In *Pr. naegleriophila*, the CRISPR locus comprises eight 28bp-long repeats separated by 33bp-long
140 spacers. The upstream operon of CRISPR-associated genes from the *E. coli* subtype I-E consists of the
141 core genes *cas1-2*, the type I gene *cas3* and subtype-specific genes *cse1-2*, *cas5*, *cas6e* and *cas7*
142 (**Figure 3**). An almost identical cas operon and a CRISPR locus were identified in *Pr. naegleriophila*
143 'Diamant' (**Figure 3**). On the contrary, this system is absent from other *Parachlamydiaceae* such as
144 strains *Pr. amoebophila* UWE25, EI2 and R18. Although a confirmed CRISPR locus is predicted by
145 CRISPRfinder (Grissa et al. 2007) in the recently released genomes of *Neochlamydia sp.* (Domman et
146 al. 2014; Ishida et al. 2014), no *cas* genes could be identified and the repeats were found to be due to
147 a highly repeated protein sequence.

148 The CRISPR spacers could give an interesting imprint of recent invasions by extrachromosomal
149 elements, but unfortunately no significant homology was found by BLASTN against the non-
150 redundant nucleotide database (nt) for strains KNic and Diamant (Sup. table 1 and sup. table 2).
151 Genes surrounding this locus are found in conserved order in all *Protochlamydia* species indicating
152 that this CRISPR region has most likely been acquired by horizontal gene transfer after the
153 divergence of *Pr. naegleriophila* from *Pr. amoebophila*. The gene operon structure is commonly
154 found in bacteria and two species present particular homology to *Pr. naegleriophila* KNic CRISPR
155 locus: *Anaeromyxobacter dehalogenans*, a *Deltaproteobacteria* from soil and *Rhodothermus marinus,*
156 a *Bacteroidetes* (**Figure 3**).

157

158 **DISCUSSION**

159 The genome sequence of *Pr. naegleriophila* presented in this contribution permitted to grasp initial
160 hints on mechanisms triggering the evolution of *Protochlamydia*. We could evidence the presence of
161 a CRISPR-locus in the chromosome for the first time in the order *Chlamydiales*. The sequencing data
162 also revealed the presence of a plasmid that encodes a type IV secretion system and is only partially
163 similar to the genomic island of *Pr. amoebophila*.

164 Amoebae were proposed to act as a reservoir of different amoebae-resisting bacteria where
165 horizontal gene transfer may preferentially take place (Moliner et al. 2010). The presence of a an F-
166 like conjugation plasmid putatively involved in DNA transfer in *Pr. naegleriophila* stresses the
167 likelihood of gene exchange with other bacteria or with the eukaryotic host. The maintenance of
168 intact *tra* genes in bacteria possessing the *tra* operon on a plasmid, suggest that the system has

bioRxiv preprint doi: https://doi.org/10.1101/028977; this version posted October 13, 2015. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

retained functionality, whereas it has evolved towards pseudogenisation and deletion after being integrated in the genome of *Pr. amoebophila* strains and *P. acanthamoebae* strains.

The presence of an F-type conjugative operon in the plasmid or in the chromosome of various strains combined with the lack of conjugative operon in the plasmid or in the chromosome of the *Waddliaceae, Criblamydiaceae* (Bertelli et al. 2015, 2014) and some *Parachlamydiaceae* challenges the most parsimonious scenario proposed by Collingro *et al* (Collingro et al. 2011) that plasmids evolved from a single conjugative plasmid acquired by an ancestor of the *Parachlamydiaceae, Waddliaceae,* and *Simkaniaceae.* In favor of this hypothesis is the shared presence of the Ti-type *traA* and *traD* in the paraphyletic *Parachlamydia sp.* Rubis, *Pr. naegleriophila* KNic, as well as *S. negevensis.* If this hypothesis is correct, the plasmid and its *tra* operon were integrated within the chromosome at least twice in the genus *Parachlamydia* and *Protochlamydia.* The *tra* operon was completely lost several times, in the families *Waddliaceae* and *Criblamydiaceae,* in the genus *Neochlamydia,* and in some strains of *Protochlamydia* and *Parachlamydia* (**Figure 2**). Furthermore, it has been partially lost in the *Parachlamydia* genus, where only a few genes remain. An alternative scenario of separate acquisition of *tra* operon by an ancestor of *Simkania* and members of the family *Parachlamydiaceae* may be envisioned. In any case, this highlights the highly dynamic nature of the *Chlamydia*-related genomes and the potential of the *tra* operon to be readily transferred, acquired, and lost among these bacteria.

A CRISPR-Cas system has been reported in approximately 50% of bacteria with sporadic distribution patterns suggesting that CRISPR loci are subject to frequent horizontal gene transfer, a hypothesis supported by the presence of CRISPR loci on plasmids (Haft et al. 2005). The CRISPR locus of *Pr. naegleriophila* and its associated genes have most probably been acquired horizontally but the proteins have insufficient homology to infer a direct transfer from a given organism. This CRISPR-Cas system is of a different subtype than that of another intracellular amoeba-resisting bacteria *F. novicida* ruling out the possibility of intra-amoebal transfer between these organisms. The functionality and the exact role of this CRISPR-Cas system in *Pr. naegleriophila* remains to be determined by laboratory experiments but by similarity to the type IE locus present in *E. coli,* we can hypothesize that it plays a role in preventing DNA acquisition or protecting against phages. Although *Pr. naegleriophila* is an obligate intracellular bacteria, it may still be exposed to phages similarly to other *Chlamydia* species (Śliwa-Dominiak et al. 2013). The difference in CRISPR spacers between *Pr. naegleriophila* strains KNic and Diamant clearly highlights the dynamic and likely functional status of the system, as well as the exposure of such obligate intracellular bacteria to DNA of foreign origin. The absence of similarity between CRISPR spacers and sequences of the non-redundant nucleotide database underlines the currently limited knowledge on phages and extrachromosomal DNA

bioRxiv preprint doi: https://doi.org/10.1101/028977; this version posted October 13, 2015. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

203 circulating in amoebae-resisting bacteria, especially those growing in the ubiquitous amoeba
204 *Naegleria.*

205 Based on the complete genome sequence of *P. acanthamoebae* UV-7 and *Pr. amoebophila* UWE25 as
206 well as four draft genomes, Dommann *et al.* (Domman et al. 2014) suggested the occurrence of few
207 rearrangements within genera of the family *Parachlamydiaceae*. Beyond the difficulty to make such
208 an assessment based on the alignment of highly fragmented draft genome sequences available in
209 public databases, the genomes used in the latter study are different strains of the same species and
210 might therefore not reflect genome evolution at the genus level. Indeed, our comparison shows the
211 absence of rearrangements between the two *Pr. naegleriophila* strains KNic and Diamant, but an
212 increasing number of genome rearrangements with further distantly-related organisms. This
213 highlights the need for complete genomes to precisely unravel the bacterial evolution occurring by
214 recombination. The complete genome sequence of *Pr. naegleriophila* represents a first step toward
215 the understanding of mechanisms triggering genome evolution and evolutionary pressures at play in
216 the *Parachlamydiaceae* family.

217

bioRxiv preprint doi: https://doi.org/10.1101/028977; this version posted October 13, 2015. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

MATERIALS AND METHODS

Culture and purification of *Pr. naegleriophila*

Protochlamydia naegleriophila strain KNic was grown in *Acanthamoeba castellanii* ATCC 30010 at 32°C using 75 cm^2 cell culture flasks (Becton Dickinson, Franklin Lakes, USA) with 30 ml of peptone-yeast extract glucose broth. *Pr. naegleriophila* were purified from amoebae by a first centrifugation step at 120 x *g* for 10 min. Then, remnants from amoebae were removed from the re-suspended bacterial pellet by centrifugation at 6500 x *g* for 30 min onto 25% sucrose (Sigma Aldrich, St Louis, USA) and finally at 32000 x *g* for 70 min onto a discontinuous Gastrographin (Bayer Schering Pharma, Zurich, Switzerland) gradient (48%/36%/28%).

Genome sequencing, assembly and gap closure

Pr. naegleriophila genomic DNA was isolated with the Wizard Genomic DNA purification kit (Promega Corporation, Madison, USA). Reads obtained with Genome Sequencer 20™ (Droege and Hill 2008) by Roche Applied Science (Penzberg, Germany) were assembled using Newbler V1.1.02.15 yielding 93 large contigs with a mean 16x coverage. Scaffolding on *Pr. amoebophila* strain UWE25 and PCR-based techniques were used to close the gaps between those contigs. Solexa 35 bp reads obtained from sequencing with Genome Analyzer GaIIx (Bennett 2004) by Fasteris (Plan les Ouates, Switzerland) were then mapped to the final assembly with BWA (Li & Durbin 2009) and visualized with Consed (Gordon & Green 2013). Homopolymer errors were corrected in the plasmid and the chromosome sequence after manual inspection of discrepancies covered by >2 reads with a phred quality score of the base >10. Sequence start was placed in an intergenic region closest to the minimum of the GC skew, as determined with a sliding window of 100nt.

Genome annotation

GenDB 2.4 pipeline (Meyer et al. 2003) was used for a first automatic annotation of the genome that was followed by manual curation of annotation. Coding sequence (CDS) prediction was performed using Prodigal (Hyatt et al. 2010). All predicted CDS were submitted to similarity searches against nr, Swissprot, InterPro, Pfam, TIGRfam and KEGG databases. Putative signal peptides, transmembrane helices and nucleic acid binding domains were predicted using respectively SignalP (Petersen et al. 2011), TMHMM (Krogh et al. 2001) and Helix-Turn-Helix (Dodd & Egan 1990). Genome annotation was manually curated with a scheme as proposed in (Bertelli et al. 2015). The complete and annotated genome sequences have been deposited in the European Nucleic Archive under the project PRJEB7990 with accession numbers LN879502 and LN879503.

bioRxiv preprint doi: https://doi.org/10.1101/028977; this version posted October 13, 2015. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Genome analysis

To identify CRISPR repeats, the genome sequences were submitted to CRISPRFinder (Grissa et al. 2007). The spacers within CRISPR locus of *Pr. naegleriophila* strains KNic and Diamant were submitted to BLASTN (Altschul et al. 1997) homology searches against the non-redundant nucleotide database. For phylogenetic reconstruction, multiple sequence alignments were performed with Muscle V3.7 (Edgar 2004), and a neighbor-joining tree was reconstructed using Mega 6 (Tamura et al. 2013) with 1000 bootstrap, poisson distribution, gamma equal to 1. The two nearly complete genomes of *Chlamydia* sp. Rubis and *Pr. naegleriophia* Diamant were reordered with Mauve (Darling et al. 2004) by similarity to the closest available complete genome sequence *P. acanthamoebae* UV7 and *Pr. naegleriophila* KNic, respectively. These genomes and the complete genomes were aligned using Mauve and the alignment was represented using GenoPlotR (Guy et al. 2010). Genomic islands were predicted using IslandViewer (Dhillon et al. 2015). Home-made scripts for data analysis and visualization were written in R (Cran 2010).

ACKNOWLEDGMENTS

We are grateful to Sébastien Aeby (University of Lausanne, Switzerland) for his technical help during the gap closure stage. We would like to thank Burkhard Linke (Justus-Liebig-University Giessen, Germany) for his assistance in maintaining this GenDB project. We acknowledge technical assistance by the Bioinformatics Core Facility at JLU Giessen and access to resources financially supported by the BMBF grant FKZ 031A533 within the de.NBI network. Part of the computations was performed at the Vital-IT (http://www.vital-it.ch) Center for high-performance computing of the SIB Swiss Institute of Bioinformatics.

bioRxiv preprint doi: https://doi.org/10.1101/028977; this version posted October 13, 2015. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

REFERENCES

Altschul SF et al. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res. 25:3389–3402.

Bertelli C et al. 2015. Sequencing and characterizing the genome of Estrella lausannensis as an undergraduate project: training students and biological insights. Front. Microbiol. 6.

Bertelli C, Goesmann A, Greub G. 2014. Criblamydia sequanensis Harbors a Megaplasmid Encoding Arsenite Resistance. Genome Announc. 2.

Bertelli C, Greub G. 2012. Lateral gene exchanges shape the genomes of amoeba-resisting microorganisms. Front. Cell. Infect. Microbiol. 2.

Bolotin A, Quinquis B, Sorokin A, Ehrlich SD. 2005. Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin. Microbiology. 151:2551–61.

Brouns SJJ et al. 2008. Small CRISPR RNAs guide antiviral defense in prokaryotes. Science. 321:960–4.

Casson N, Michel R, Müller K-D, Aubert JD, Greub G. 2008. Protochlamydia naegleriophila as etiologic agent of pneumonia. Emerg. Infect. Dis. 14:168–72.

Collingro A et al. 2011. Unity in Variety - the Pan-Genome of the Chlamydiae. Mol. Biol. Evol. 28:3253–3270.

Cran. 2010. The Comprehensive R Archive Network. Wiley Interdiscip. Rev. Comput. Stat. n/a–n/a.

Darby AC, Cho N-H, Fuxelius H-H, Westberg J, Andersson SGE. 2007. Intracellular pathogens go extreme: genome evolution in the Rickettsiales. Trends Genet. 23:511–20.

Darling ACE, Mau B, Blattner FR, Perna NT. 2004. Mauve: multiple alignment of conserved genomic sequence with rearrangements. Genome Res. 14:1394–1403.

Deveau H, Garneau JE, Moineau S. 2010. CRISPR/Cas system and its role in phage-bacteria interactions. Annu. Rev. Microbiol. 64:475–93.

Dhillon BK et al. 2015. IslandViewer 3: more flexible, interactive genomic island discovery, visualization and analysis. Nucleic Acids Res. 43:W104–8.

Dodd IB, Egan JB. 1990. Improved detection of helix-turn-helix DNA-binding motifs in protein sequences. Nucleic Acids Res. 18:5019–26.

Domman D et al. 2014. Massive expansion of ubiquitination-related gene families within the Chlamydiae. Mol. Biol. Evol.

Edgar RC. 2004. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics. 5:113.

Eugster M, Roten C-AH, Greub G. 2007. Analyses of six homologous proteins of Protochlamydia amoebophila UWE25 encoded by large GC-rich genes (lgr): a model of evolution and concatenation of leucine-rich repeats. BMC Evol. Biol. 7:231.

bioRxiv preprint doi: https://doi.org/10.1101/028977; this version posted October 13, 2015. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

307 Everett KDE, Bush RM, Andersen AA. 1999. Emended description of the order Chlamydiales, proposal
308 of Parachlamydiaceae fam. nov. and Simkaniaceae fam. nov., each containing one monotypic genus,
309 revised taxonomy of the family Chlamydiaceae, including a new genus and five new species, and
310 standards. Int. J. Syst. Bacteriol. 49:415–440.

311 Gimenez G et al. 2011. Insight into cross-talk between intra-amoebal pathogens. BMC Genomics.
312 12:542.

313 Gordon D, Green P. 2013. Consed: a graphical editor for next-generation sequencing. Bioinformatics.
314 29:2936–7.

315 Greub G et al. 2009. High throughput sequencing and Proteomics to identify immunogenic proteins
316 of a new pathogen: The dirty genome approach. PLoS One. 4.

317 Greub G. 2010. International Committee on Systematics of Prokaryotes * Subcommittee on the
318 taxonomy of the Chlamydiae: Minutes of the inaugural closed meeting, 21 March 2009, Little Rock,
319 AR, USA. Int. J. Syst. Evol. Microbiol. 60:2691–2693.

320 Greub G. 2009. Parachlamydia acanthamoebae, an emerging agent of pneumonia. Clin. Microbiol.
321 Infect. 15:18–28.

322 Greub G, Collyn F, Guy L, Roten C-A. 2004. A genomic island present along the bacterial chromosome
323 of the Parachlamydiaceae UWE25, an obligate amoebal endosymbiont, encodes a potentially
324 functional F-like conjugative DNA transfer system. BMC Microbiol. 4:48.

325 Grissa I, Vergnaud G, Pourcel C. 2007. CRISPRFinder: a web tool to identify clustered regularly
326 interspaced short palindromic repeats. Nucleic Acids Res. 35:W52–7.

327 Guy L, Kultima JR, Andersson SGE. 2010. genoPlotR: comparative gene and genome visualization in R.
328 Bioinformatics. 26:2334–5.

329 Haft DH, Selengut J, Mongodin EF, Nelson KE. 2005. A guild of 45 CRISPR-associated (Cas) protein
330 families and multiple CRISPR/Cas subtypes exist in prokaryotic genomes. PLoS Comput. Biol. 1:e60.

331 Horn M et al. 2004. Illuminating the evolutionary history of chlamydiae. Science. 304:728–730.

332 Horn M. 2011. Phylum XXIV. Chlamydiae Garrity and Holt 2001. In: Bergey's Manual of Systematic
333 Bacteriology - Volume 4: The | Noel R. Krieg | Springer. Krieg, NR et al., editors. Springer Berlin
334 Heidelberg.

335 Horvath P, Barrangou R. 2010. CRISPR/Cas, the immune system of bacteria and archaea. Science.
336 327:167–70.

337 Hyatt D et al. 2010. Prodigal: prokaryotic gene recognition and translation initiation site
338 identification. BMC Bioinformatics. 11:119.

339 Ishida K et al. 2014. Amoebal endosymbiont Neochlamydia genome sequence illuminates the
340 bacterial role in the defense of the host amoebae against Legionella pneumophila. PLoS One. 9.

bioRxiv preprint doi: https://doi.org/10.1101/028977; this version posted October 13, 2015. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

341 Ishino Y, Shinagawa H, Makino K, Amemura M, Nakata A. 1987. Nucleotide sequence of the iap gene,
342 responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the
343 gene product. J. Bacteriol. 169:5429–33.

344 Jansen R, Embden JDA van, Gaastra W, Schouls LM. 2002. Identification of genes that are associated
345 with DNA repeats in prokaryotes. Mol. Microbiol. 43:1565–75.

346 Kamneva OK, Knight SJ, Liberles DA, Ward NL. 2012. Analysis of genome content evolution in PVC
347 bacterial super-phylum: Assessment of candidate genes associated with cellular organization and
348 lifestyle. Genome Biol. Evol. 4:1375–1380.

349 Koonin E V, Makarova KS. 2013. CRISPR-Cas: evolution of an RNA-based adaptive immunity system in
350 prokaryotes. RNA Biol. 10:679–86.

351 Krogh A, Larsson B, von Heijne G, Sonnhammer EL. 2001. Predicting transmembrane protein topology
352 with a hidden Markov model: application to complete genomes. J. Mol. Biol. 305:567–80.

353 Lagkouvardos I et al. 2014. Integrating metagenomic and amplicon databases to resolve the
354 phylogenetic and ecological diversity of the Chlamydiae. ISME J. 8:115–25.

355 Li H, Durbin R. 2009. Fast and accurate short read alignment with Burrows-Wheeler transform.
356 Bioinformatics. 25:1754–1760.

357 Louwen R, Staals RHJ, Endtz HP, van Baarlen P, van der Oost J. 2014. The role of CRISPR-Cas systems
358 in virulence of pathogenic bacteria. Microbiol. Mol. Biol. Rev. 78:74–88.

359 Makarova KS et al. 2011. Evolution and classification of the CRISPR-Cas systems. Nat. Rev. Microbiol.
360 9:467–77.

361 Meyer F et al. 2003. GenDB--an open source genome annotation system for prokaryote genomes.
362 Nucleic Acids Res. 31:2187–2195.

363 Moliner C, Fournier P-E, Raoult D. 2010. Genome analysis of microorganisms living in amoebae
364 reveals a melting pot of evolution. FEMS Microbiol. Rev. 34:281–94.

365 Petersen TN, Brunak S, von Heijne G, Nielsen H. 2011. SignalP 4.0: discriminating signal peptides from
366 transmembrane regions. Nat. Methods. 8:785–6.

367 Sampson TR, Weiss DS. 2014. CRISPR-Cas systems: new players in gene regulation and bacterial
368 physiology. Front. Cell. Infect. Microbiol. 4:37.

369 Śliwa-Dominiak J, Suszyńska E, Pawlikowska M, Deptuła W. 2013. Chlamydia bacteriophages. Arch.
370 Microbiol. 195:765–771.

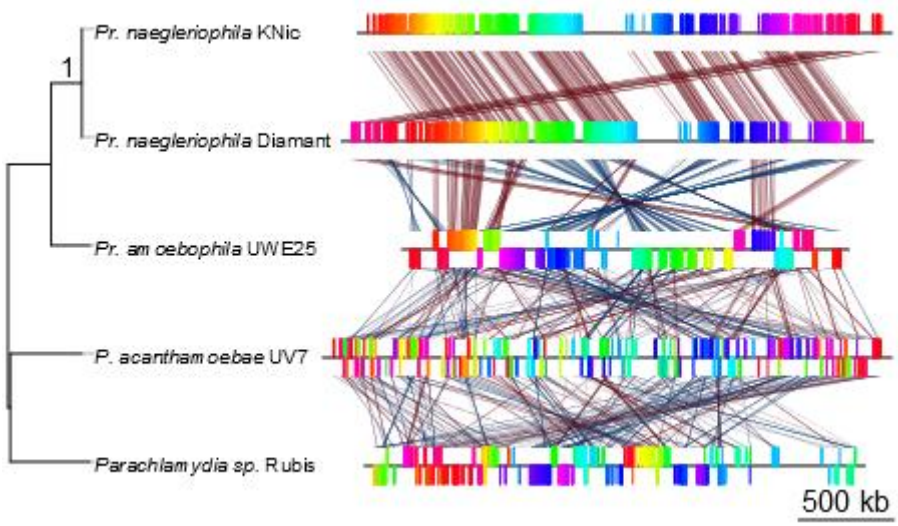
371 Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics
372 Analysis version 6.0. Mol. Biol. Evol. 30:2725–9.

373 Weinberger AD, Wolf YI, Lobkovsky AE, Gilmore MS, Koonin E V. 2012. Viral diversity threshold for
374 adaptive immunity in prokaryotes. MBio. 3:e00456–12.

375

bioRxiv preprint doi: https://doi.org/10.1101/028977; this version posted October 13, 2015. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

376 **FIGURES**



377

378 **Figure 1. Genomic rearrangements in the *Parachlamydiaceae* family**

379 Left side, the phylogenetic branching of bacterial strains as inferred by a Neighbor-Joining tree
380 reconstruction based on 5 conserved proteins (DnaA, FtsK, HemL, FabI and SucA). Right side,
381 visualization of genomic rearrangements in the family *Parachlamydiaceae*. The two strains of the
382 species *Protochlamydia naegleriophila* are highly collinear, with no apparent rearrangement other
383 than due to the choice of the start of the genome sequence. With increasing distances between
384 organisms, the genomes show increasing number of rearrangements.

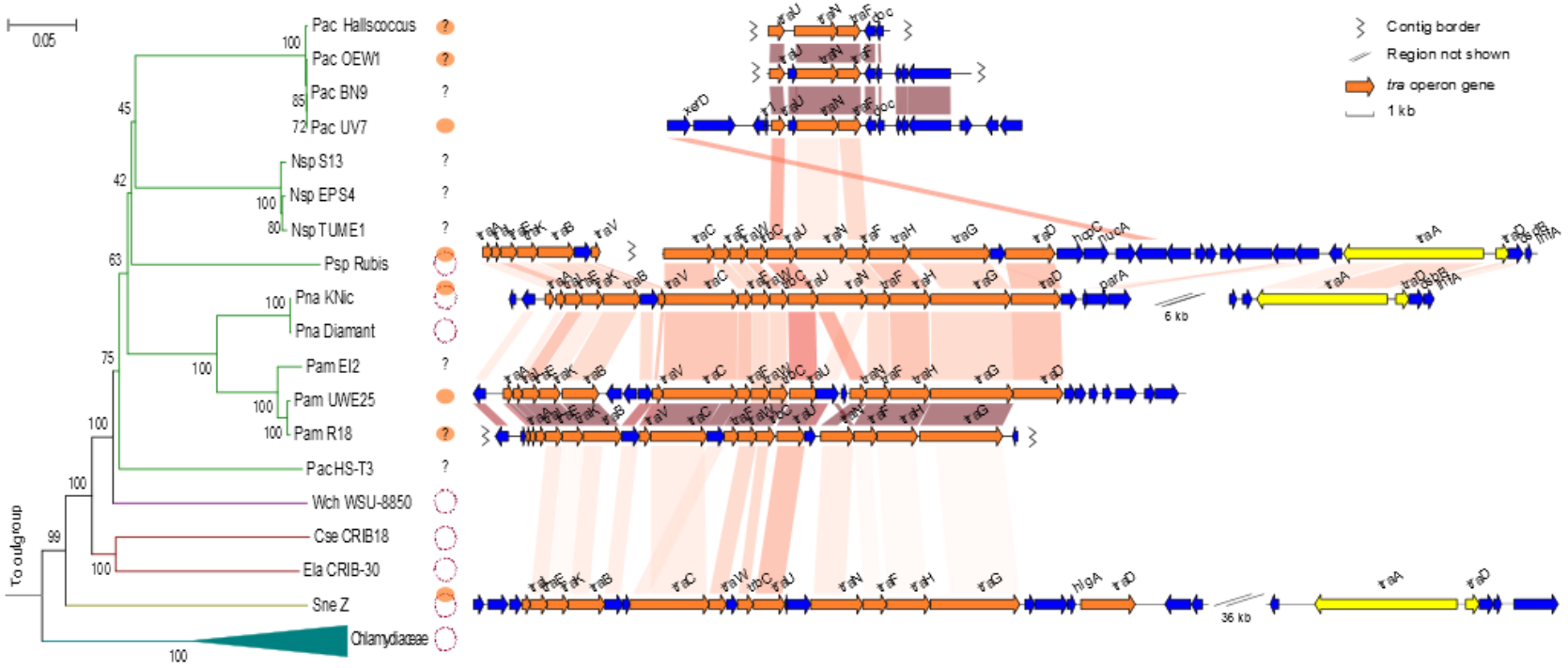
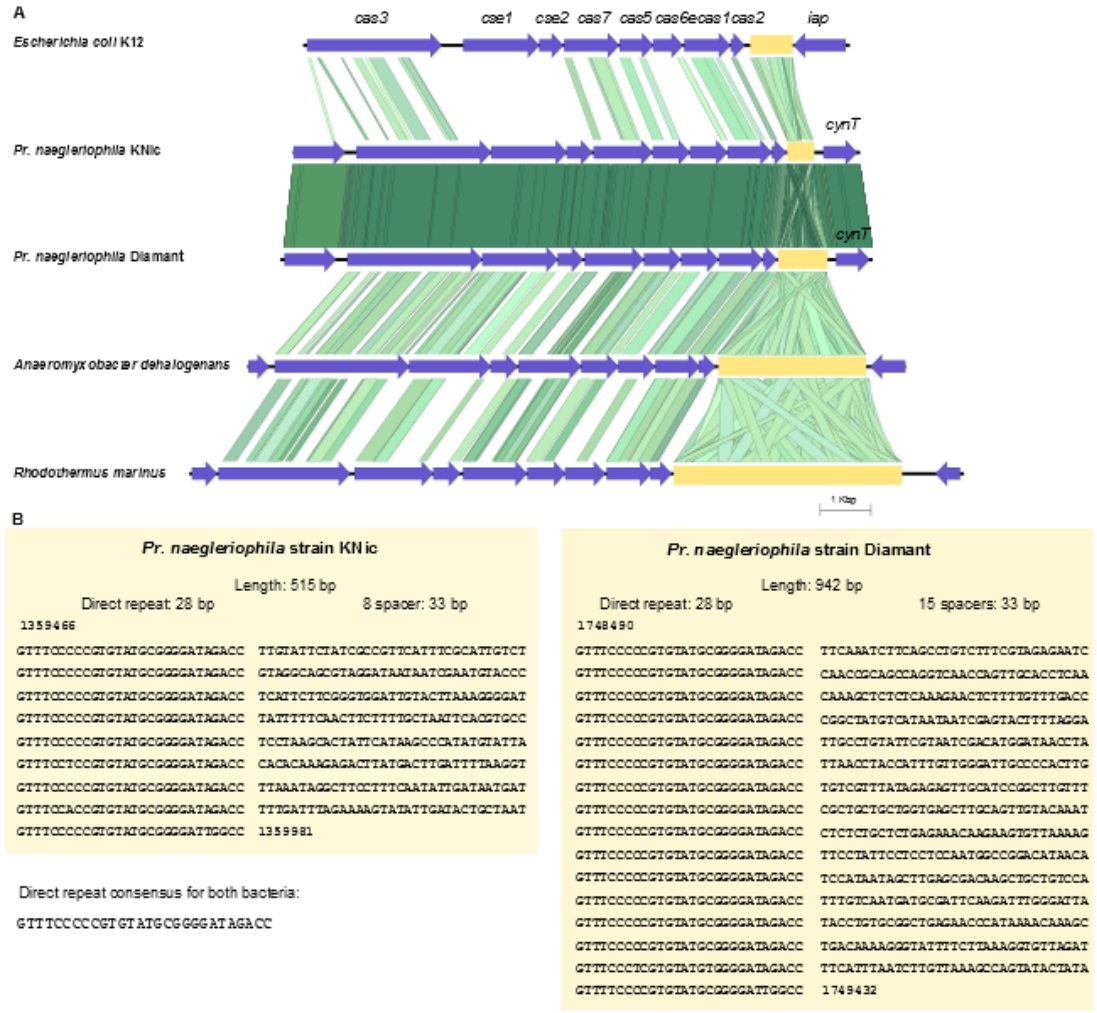


Figure 2. *Chlamydiales* order, plasmids and type IV secretion system

The left panel represents a neighbor joining tree of bacteria belonging the order *Chlamydiales* whose genome sequences is available based on four conserved proteins (DnaA, FtsK, HemL, FabI). The presence of a plasmid in each strain is represented by a small circular DNA molecule, and the draft genomes with no known plasmid described are indicated by a question mark as plasmids may be hidden among the numerous contigs. Orange ovals indicate the presence of a conjugative *tra* operon on the plasmid or in the bacterial chromosome. The right panel shows the conservation of the type IV secretion system *tra* operon and the surrounding genes. Pac: *P. acanthamoebae,* Nsp: *Neochlamydia* sp., Psp*: Protochlamydia sp.,* Pna: *Pr. naegleriophila,* Pam: *Pr. amoebophila,* PacHS-T3: *Parachlamydiaceae* bacterium HS-T3.

bioRxiv preprint doi: https://doi.org/10.1101/028977; this version posted October 13, 2015. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.



393

394 **Figure 3. CRISPR locus and its associated genes**

395 A. CRISPR associated genes (CAS) consist of eight coding sequences, *cas3, cse1, cse2, cas7, cas5,*
396 *cas6e*, *cas1* and *cas2*, shown in blue within their genomic environment. Green lines connecting the
397 genes in different organisms represent BLAST sequence homology with a gradient from light green to
398 dark green for low to high percentage sequence identity, respectively. Genes neighboring the CRISPR
399 locus present homology in *Pr. naegleriophila* genomes, but not to other genomes showing that the
400 site of CRISPR locus insertion in *Pr. naegleriophila* genomes is different than in other bacteria. CRISPR
401 repeats are found directly downstream of the CAS operon, as highlighted by the yellow box. B. Direct
402 repeats and spacer sequences are detailed below.

403

404 **TABLES**

Species	Strain	Status	Scaffolds	Genome size	GC content	% coding	CDS	tRNAs	rRNA genes	Plasmid size	Plasmid CDS	Plasmid GC content
Protochlamydia amoebophila	**UWE25**	Complete	1	2,414,465	34.7		1855	35	7	-		
Protochlamydia amoebophila	**EI2**	Draft	178	2,397,675	34.8		1797	36	3	NA		
Protochlamydia amoebophila	**R18**	Draft	795	2,881,499	34.8		2025	41	13	NA		
Protochlamydia naegleriophila	**KNic**	Complete	1	2,885,090	42.7		2415	43	12	145,285	160	37.2
Protochlamydia naegleriophila	**Diamant**	Draft	4*	2,864,073	42.8		2424	39	7	91,928	98	40.9
Parachlamydia acanthamoebae	**UV7**	Complete	1	3,072,383	39		2531	40	10	-		
Parachlamydia acanthamoebae	**Hall's coccus**	Draft	95	2,971,261	39		2474	35	3	NA		
Parachlamydia acanthamoebae	**OEW1**	Draft	162	3,008,885	39		2321	38	4	NA		
Parachlamydia acanthamoebae	**Bn9**	Draft	72	2,999,361	38.9		2498°	NA	NA	NA		
Parachlamydiaceae **bacterium**	**HS-T3**	Draft	34	2,307,885	38.7		2003	39	3	NA		
Parachlamydia sp.	**Rubis**	Draft	3*	2,701,449	32.4		2446	36	5	80,697°	107	40.2
										39,075°	40	29.8
Neochlamydia sp.	**EPS4**	Draft	112	2,530,677	38.1		1843	36	4	NA		
Neochlamydia sp.	**TUME1**	Draft	254	2,546,323	38		1834	36	4	NA		
Neochlamydia sp.	**S13**	Draft	1342	3,187,074	38		2175	42	10	NA		

*** Following removal of the plasmid(s) present according to our analyses. ° Not circularized, estimated size . NA : information not available**

405

406 **Table 1. Genomics characteristics of bacteria belonging to the family *Parachlamydiaceae***

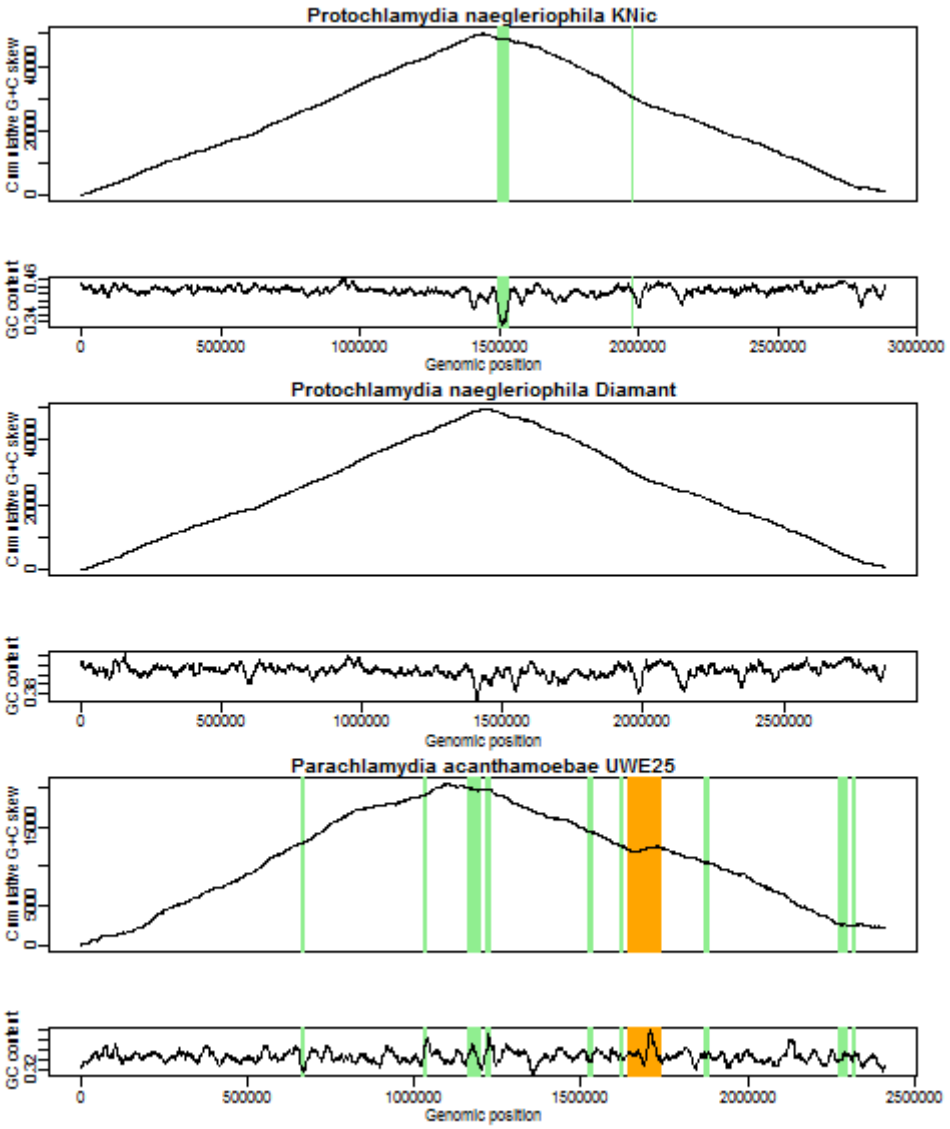
407 As available on NCBI database on 22.09.2015, all genomes except KNic have been reannotated by the NCBI Prokaryotic Genome Annotation Pipeline.

408

409

bioRxiv preprint doi: https://doi.org/10.1101/028977; this version posted October 13, 2015. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

410 **SUPPLEMENTARY FIGURES**



411

412 **Figure S1. Nucleotide biases and genomic islands**

413 The GC content and the bias of G versus C (GC skew) were determined in a sliding window of 1000 bp
414 along the genome sequence of *Pr. naegleriophila* KNic, *Pr. naegleriophila* Diamant and *Pr.*
415 *amoebophila* UWE25. The maximum of the GC skew indicates the putative terminus of replication.
416 The origin of replication is found at the minimum of the curve and has been used to assess the first
417 base of the genomic sequences. The contigs of *Pr. naegleriophila* Diamant have been reordered by
418 similarity to strain KNic and the putative plasmid removed. The location of predicted GIs for *Pr.*
419 *naegleriophila* KNic and *Pr amoebophila* UWE25 is indicated in green, whereas the confirmed *Pr.*
420 *amoebophila* genomic island Pam100G is indicated in orange.

bioRxiv preprint doi: https://doi.org/10.1101/028977; this version posted October 13, 2015. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

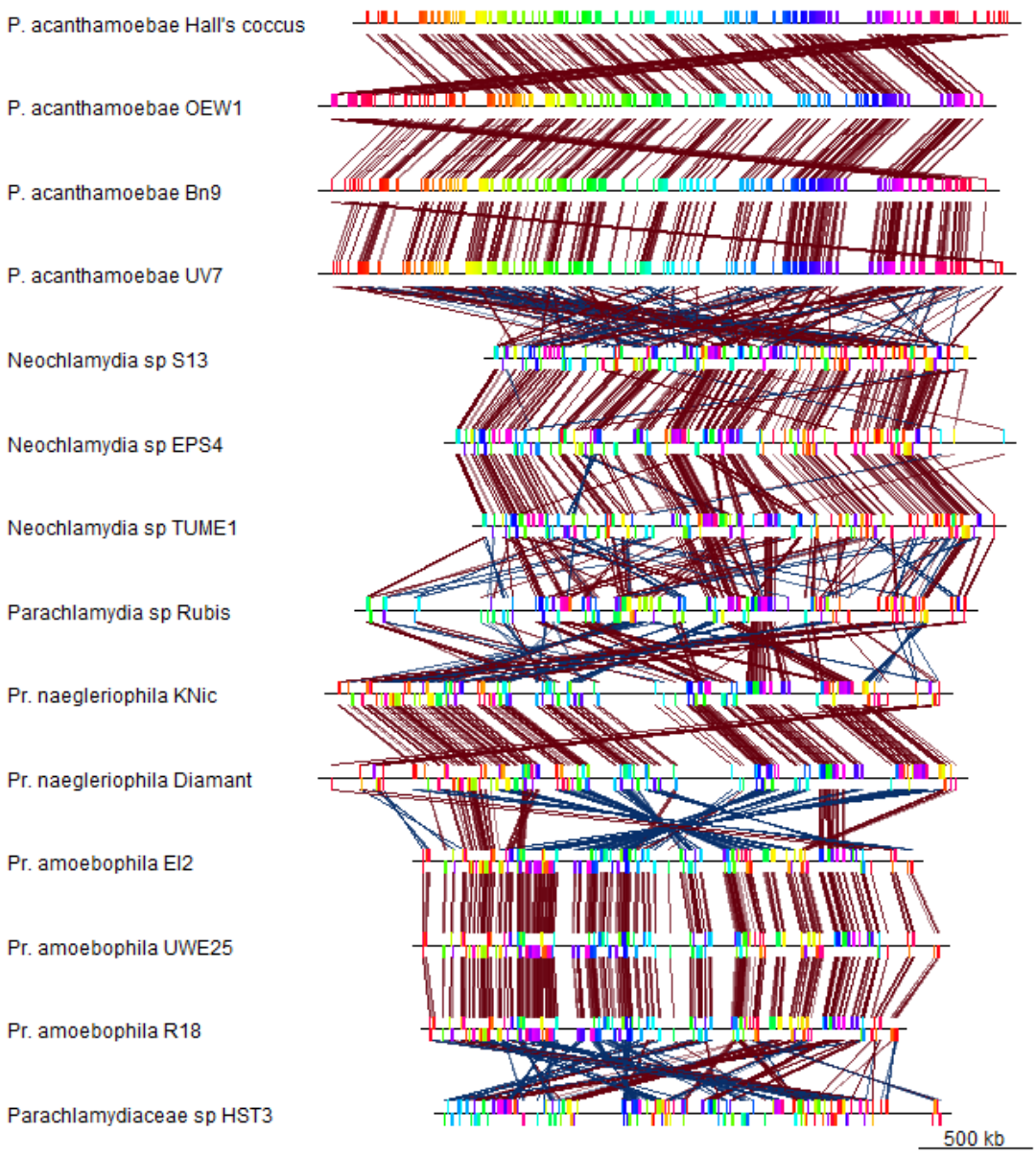


Figure S2. Genome rearrangements in the *Parachlamydiaceae*

This figure shows the difficulty of using draft genomes to observe genomic rearrangements. Available draft genomes of members of the family *Parachlamydiaceae* were reordered using Mauve against the complete genome of the most closely-related organism: *Pr. naegleriophila* Diamant and *Chlamydia* sp. Rubis against *Pr. naegleriophila* KNic; *Pr. amoebophila* EI2 and R18 against *Pr. amoebophila* UWE25; *P. acanthamoebae* BN9, Hall's coccus, and OEW1 against *P. acanthamoebae* UV7; and *Parachlamydia* sp. HST3 and *Neochlamydia* sp. S13 against *Chlamydia* sp. Rubis, and *Neochlamydia* sp. EPS4 and TUME1 against *Neochlamydia* sp. S13. As expected, no rearrangement can be observed after the rearrangement of a fragmented draft genome against closely-related strain of the same species. In the case of *Neochlamydia* sp. S13 and *Parachlamydia* sp. HST3 for which no complete genome is available, numerous rearrangements can be seen, most likely as an artifact due to the reduced similarity at nucleotide level between the reordered genome and the reference genome.

bioRxiv preprint doi: https://doi.org/10.1101/028977; this version posted October 13, 2015. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

435 **SUPPLEMENTARY TABLES**

436

Query	Percentage identity	Alignment length	Query coverage	Organism
spacer_1	95.83	24	73	Nippostrongylus brasiliensis (nematoda)
spacer_2	100	19	58	Sphingobacterium sp. PM2-P1-29 (bacteroides)
spacer_3	100	20	61	Paenibacillus polymyxa CR1 (firmicutes)
spacer_4	93.1	29	88	Tinamus guttatus (bird)
spacer_5	95.65	23	70	Sus scrofa (mammal)
spacer_6	92.86	28	85	Schistosoma margrebowiei (platyhelmintes)
spacer_7	85.71	35	97	Alteromonas macleodii str. 'Black Sea 11‘ (γ-prot)
spacer_8	93.1	29	88	Solanum pennellii (plant)

437

438 **Table S1 Homology of *P. naegleriophila* KNic spacers against the non-redundant nucleotide**
439 **database by BLASTN**

440

Query	Percentage identity	Alignment length	Query coverage	Organism
spacer_1	100	19	58	uncultured archaeon
spacer_2	100	21	64	Pseudomonas aeruginosa
spacer_3	90	30	91	Populus euphratica
spacer_4	89.29	28	85	Spirometra erinaceieuropaei
spacer_5	92	25	76	Toxocara canis
spacer_6	95.65	23	70	Xenorhabdus nematophila AN6/1
spacer_7	100	19	58	Heterobasidion irregulare TC 32-1
spacer_8	87.5	32	97	Kluyveromyces marxianus
spacer_9	96	25	76	Homo sapiens
spacer_10	95.83	24	73	Phoenix dactylifera
spacer_10	100	21	64	Candidatus Nitrososphaera evergladensis SR1
spacer_11	95.83	24	73	Solanum tuberosum
spacer_12	96.3	27	82	Staphylococcus aureus subsp. aureus MSHR1132
spacer_13	100	21	64	Vibrio fischeri MJ11
spacer_14	92.31	26	79	Borrelia garinii PBi
spacer_15	92.59	27	82	Echinostoma caproni

441

442 **Table S2 Homology of *P. naegleriophila* Diamant spacers against the non-redundant nucleotide**
443 **database by BLASTN**

444