Missing links in the divergence of *Chlamydophila abortus* from *Chlamydophila psittaci*

M. Van Loock,¹ D. Vanrompay,² B. Herrmann,³ J. Vander Stappen,¹
G. Volckaert,¹ B. M. Goddeeris¹ and K. D. E. Everett⁴

Correspondence

D. Vanrompay
Daisy.Vanrompay@rug.ac.be

¹Department of Animal Sciences, Katholieke Universiteit Leuven, Kasteelpark Arenberg 21 (Laboratory of Gene Technology) and 30 (Laboratory of Physiology and Immunology of Domestic Animals), 3001 Leuven, Belgium

²Department of Molecular Biotechnology, Ghent University, Coupure Links 653, 9000 Ghent, Belgium

³Department of Clinical Microbiology, University Hospital, S-751 85 Uppsala, Sweden

⁴Department of Microbiology, University of Georgia, Athens, GA 30602, USA

Pathological and serological evidence and DNA–DNA reassociation data indicate that *Chlamydophila psittaci* and *Chlamydophila abortus* are separate species. *C. psittaci* causes avian systemic disease and *C. abortus* causes abortion. Both previously belonged to *Chlamydia psittaci* are associated with zoonotic and enzootic outbreaks. Genetic studies suggest that they are closely related and because of the recent availability of diverse *C. psittaci* strains and comparative data for several genes, it was possible to explore this relationship. The parrot *C. psittaci* strain 84/2334 was found to have DNA sequences that were identical to an extrachromosomal plasmid in duck *C. psittaci* strain N352, to *rnpB* in strain R54 from a brown skua and to the *rrn* intergenic spacer in parakeet strain Prk/Daruma (from Germany, Antarctica and Japan, respectively). Analysis of *ompA* and the *rrn* spacer revealed progressive diversification of the strains, with 84/2334 resembling what might have been a recent ancestor of *C. abortus*. Another *C. psittaci* strain (VS225) showed evidence of having undergone convergent evolution towards the *C. abortus*-like genotype, whereas strain R54 diverged independently. For the first time, these studies link *C. abortus* in an evolutionary context to the *C. psittaci* lineage. It has been concluded that *C. abortus* diverged from *C. psittaci*, and so strain R54 was designated a *C. psittaci* strain. It is recommended that characterization of *C. psittaci* and *C. abortus* strains should utilize more than a single method and more than a single gene.

INTRODUCTION

Chlamydophila psittaci is a lethal intracellular bacterial species that causes avian chlamydiosis, epizootic outbreaks in mammals and respiratory psittacosis in humans (Durfee *et al.*, 1975; Anderson *et al.*, 1978; Edwards, 1981; Hedberg *et al.*, 1989; Ni *et al.*, 1996; Brewis & McFerran, 1997; Goupil *et al.*, 1998; Bennedsen & Filskov, 2000; Centers for Disease Control and Prevention, 2000). *Chlamydophila abortus*

causes abortion and foetal death in mammals, including humans (Wong *et al.*, 1985; Jorgensen, 1997; Rodolakis *et al.*, 1998). In the absence of sequence data, these species have no apparent relationship. Side-by-side comparisons of *C. psittaci* and *C. abortus* in birds and other animals show differences in infectivity, persistence, disease and seroconversion (Page, 1966; Johnson & Grimes, 1983; Tappe *et al.*, 1989). Different mAbs recognize *C. psittaci* and *C. abortus* (Perez-Martinez & Storz, 1985; Eb *et al.*, 1986; Fukushi *et al.*, 1987; Takahashi *et al.*, 1988; Andersen & Van Deusen, 1988; Kikuta *et al.*, 1991; Andersen, 1991; Vretou *et al.*, 1996). *C. psittaci* and *C. abortus* genomes exhibit different restriction endonuclease patterns (Herring *et al.*, 1987; Timms *et al.*, 1988; McClenaghan *et al.*, 1991; Sayada *et al.*, 1995). DNA–DNA reassociation studies show that *C. psittaci* and *C. abortus* are 27–85 % similar (Cox *et al.*, 1988; Fukushi & Hirai, 1989). *C. psittaci* strains more often than not have an extrachromosomal plasmid, while *C. abortus* strains have no plasmid (Everett *et al.*, 1999). The genetic criteria responsible for the differing pathogenicity of *C. psittaci* and

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Abbreviations: CS, conserved segment; MOMP, major outer-membrane protein; NJ, neighbour-joining; SNP, single nucleotide polymorphism; VS, variable segment.

The GenBank accession numbers for the sequences obtained in this study are AJ310735–AJ310737 and AF481048–AF481052, as detailed in Figs 2 and 3.

The sequence alignments used to generate the phylograms shown in Figs 2 and 3 are available as supplementary material in IJSEM Online.

C. abortus are not known, and the selective pressures that drive the separation of these species are not understood.

Isolates that do not conform to this picture have been described and in some cases, the inconsistencies have been resolved. For example, enzootic abortion of ewes (EAE) strain A22/M, which had characteristics of *C. psittaci*, was shown to be a culture contaminant when bona fide *C. abortus* A22 was sequenced (Bush & Everett, 2001). *C. psittaci* strain M56 was reported to have a serotype characteristic of *Chlamydophila felis*, using polyclonal sera (Fukushi & Hirai, 1988); M56 proved to be a mixed culture (Herrmann *et al.*, 2000a). In other cases, inconsistencies have not been resolved. For example, strain R54, identified in a bird that was captured in the Antarctic, appeared to be related more closely to *C. abortus* than to *C. psittaci* (Herrmann *et al.*, 2000b). *C. psittaci* strain Prk/Daruma was reported to have a *C. felis* serotype, using polyclonal sera (Fukushi & Hirai, 1988), but it was also recognized by avian mAbs (Fukushi *et al.*, 1987). DNA–DNA reassociation data for Prk/Daruma were inconclusive (Fukushi & Hirai, 1989). Analysis of Prk/Daruma 16S rRNA shows that it is intermediate between those of *C. psittaci* and *C. abortus* (Pudjiatmoko *et al.*, 1997). Prk/Daruma has a typical *C. psittaci* restriction enzyme ribosomal profile and a unique *ompA* *AluI* restriction enzyme profile (genotype F) (Fukushi *et al.*, 1987; Fukushi & Hirai, 1988; Sayada *et al.*, 1995; Everett & Andersen, 1999). The genotype F profile was also found in *C. psittaci* strains 10433-MA (Sayada *et al.*, 1995)

and 84/2334 (Vanrompay *et al.*, 1997). Strain 84/2334 is recognized by serovar A mAbs, the same serotyping mAbs that recognize the *C. psittaci* type strain 6BC^T and a large proportion of other *C. psittaci* isolates (Vanrompay *et al.*, 1997).

Despite obvious pathological differences, analysis of gene sequences shows that *C. psittaci* comprises an unresolved cluster of strains, from which *C. abortus* is evolving (Bush & Everett, 2001). To clarify these relationships further, this study characterized genes from six *C. psittaci* isolates (84/2334, VS225, VS1, Prk/Daruma, MNRh and MNOs). These genes included *ompA*, *rnpB* and the *rrn* intergenic spacer. Furthermore, the presence of an extrachromosomal plasmid was investigated. Analysis and comparison of these genetic loci with those of other *Chlamydophila* strains, including *C. abortus*, reconciled inconsistencies in the existing data and allowed us to test the hypothesis that *C. abortus* is evolving from *C. psittaci*. These findings clarify the distinction of these two species and highlight the importance of identifying the genetic elements responsible for *C. psittaci* pathogenicity.

METHODS

Strains and DNA preparation. Strains used in this study are shown in Table 1. Isolation and growth of *C. psittaci* strain 84/2334 was performed as described previously (Vanrompay *et al.*, 1992, 1997). DNA templates were prepared as described previously (Everett *et al.*, 1991; Herrmann *et al.*, 2000a). DNA from strain

Table 1. Strains and sources

Sources of strains are abbreviated to: J. W. Frost, Staatliches Medizinal-Lebensmittel und Veterinär Untersuchungsamt, Frankfurt am Main, Germany; APHIS, Animal and Plant Health Inspection Service, Ames, IA, USA; NADC, ARS–USDA National Animal Disease Center, Ames, IA, USA; J. Storz, Veterinary School of Medicine, Louisiana State University, LA, USA. The Storz strains were abortifacient.

Strain	Serotype	Host	Tissue	Origin	Provided by
<i>C. psittaci</i>					
84/2334	A	Imported yellow-headed Amazon parrot	Lungs	Germany, 1984	J. W. Frost
VS1	A	Parrot	–	Georgia, USA, 1985	APHIS
VS225	–	Orange-fronted parakeet	–	Laredo, TX, USA, 1991	APHIS
Prk/Daruma	–	Parakeet	Systemic	Japan	H. Fukushi
MNOs	E	Ostrich	–	Texas, USA, 1993	Andersen <i>et al.</i> (1998); NADC
MNRh	E	Rhea	–	Arizona, USA, 1993	Andersen <i>et al.</i> (1998); NADC
6BC ^T	A	Parakeet	Systemic	California, USA, 1941	Everett & Andersen (1997); NADC
<i>C. abortus</i>					
B577 ^T	1	Sheep	–	Idaho, USA, 1962	Everett & Andersen (1997); NADC
GP1 NGP-17/11-14	–	Guinea pig	–	Louisiana, USA, 1979	J. Storz
GP3 NPG-LX 603C-12	–	Guinea pig	–	Louisiana, USA, 1970	J. Storz
M1 Bab-mouse-5	–	Mouse	–	Louisiana, USA, 1976	J. Storz
R1 PNT-7 Montana rabbit	–	Rabbit	–	Montana, USA, 1976	J. Storz

84/2334 was obtained using the Qiagen genomic DNA purification procedure. Extracts of a possible extrachromosomal plasmid were obtained from approximately 10^8 inclusion-forming units for each of strains 84/2334, 6BC^T and B577^T using the Qiagen TIP 500 plasmid midi kit.

PCR, electrophoresis and cloning. Chlamydial extrachromosomal plasmid eluates were subjected to PCR amplification using primers matching the highly conserved ORF8–ORF1 region in *C. psittaci* and *Chlamydomophila pneumoniae* plasmids [GenBank accession nos M32753 (Hugall *et al.*, 1989), X62475 (M. E. Lusher, J. Gregory, C. C. Storey & S. J. Richmond, unpublished) and X82078 (Thomas *et al.*, 1997)] (Table 2). Eluates were PCR-amplified in 50 mM KCl, 20 mM Tris/HCl (pH 8.3), 2 mM MgCl₂, 0.1 % Tween 20, 200 µM each dNTP, 20 µM each primer and 0.1 U SuperTaq polymerase (5 U µl⁻¹). After an initial denaturation at 95 °C for 5 min, 30 cycles of 1 min at 95 °C, 2 min at 52 °C and 3 min at 72 °C were performed, with a final extension at 72 °C for 5 min. PCR products were detected after electrophoresis on a 1.2 % agarose gel by using ethidium bromide staining and UV illumination. A CELIF dsDNA 100 bp ladder (Bio-Rad) was used as a marker.

Three chromosomal templates were targeted by a PCR: *ompA*, *rnpB* and the *rrn* spacer. Primers, amplification and sequencing protocols for these segments have been described previously (Table 2). The *C. psittaci* strain 84/2334 *ompA* PCR product was cloned into pGEM-T (Promega), and clones with inserts were selected on Luria–Bertani/ampicillin/IPTG/X-Gal plates. White colonies were tested for insert size by PCR amplification using universal T7 and SP6 primers, and six clones that produced PCR products of approximately 1100 bp were selected for sequence characterization. Initial analysis of these clones indicated that they were apparently identical, so one was selected randomly for full-length sequencing.

Sequence analyses. The sequence of the *C. psittaci* 84/2334 *ompA* clone was determined by using T7, SP6 and additional primers. The primers given in Table 2 were used for direct sequencing of the extrachromosomal plasmid, *rrn* spacer and *rnpB* PCR products after removal of amplification primers by filtration (Microcon 100 micro-concentrators; Millipore). The dideoxynucleotide chain-termination method (Sanger *et al.*, 1977) and Big Dye PCR cycle sequencing were used with ABI automated DNA sequencers (Applied Biosystems) to obtain sequence data from clones and from PCR products. All PCR products were sequenced on both strands.

Alignments and phylogenetic analyses. The sequences of *ompA*, *rnpB* and the *rrn* spacer and the deduced amino acid sequence of the major outer-membrane protein (MOMP) were aligned with related sequences identified by using BLAST (<http://www.ncbi.nlm.nih.gov>). Multiple alignment was done with CLUSTAL X software (default settings) (Thompson *et al.*, 1997) followed by manual editing; gapped

positions were removed. Neighbour-joining (NJ) analysis was conducted with PAUP* 4.0b8 (Swofford, 2001) using heuristic searching to construct a 50 % majority-rule consensus tree for both the *ompA* gene and the intergenic spacer (100 replications, ACCTRAN character optimization, TBR + MULTREES branch-swapping option). Jukes–Cantor pairwise corrected distances were calculated for the *ompA* gene and the *rrn* spacer (Jukes & Cantor, 1969). Branching-order reliability was evaluated by 100 replications of bootstrap resampling. The sequence alignments are available as supplementary material in IJSEM Online.

RESULTS

C. psittaci strain 84/2334 has an extrachromosomal plasmid

Using primers specific to the *C. psittaci* extrachromosomal plasmid, plasmid segments could be amplified by PCR from both *C. psittaci* strains 84/2334 and 6BC^T (Fig. 1a). An extrachromosomal plasmid is often present in *C. psittaci* strains, but has not been found in *C. abortus*. This PCR did not generate a product from the *C. abortus* B577^T template (Fig. 1a). Sequence analysis of the strain 84/2334 PCR product revealed that it was 469 bp long (Fig. 1b) and identical to a GenBank sequence for the *C. psittaci* extrachromosomal plasmid pCpA1 (strain N352, accession no. X62475). The 84/2334 sequence differed in four positions from the *C. psittaci* pAP'p plasmid sequence (accession no. M32753). The plasmid segments from 84/2334 and pCpA1 differed by approximately 25 % from the *Chlamydomophila caviae* plasmid sequence (<http://www.tigr.org/>), 31 % from the *Chlamydomophila pecorum* Koala II plasmid, 32 % from the *C. pneumoniae* N16 plasmid and 47 % from plasmids in *Chlamydia trachomatis* LGV440 and *Chlamydia muridarum* MoPn. This indicated that the *C. psittaci*-like extrachromosomal plasmid was present in strain 84/2334.

rRNA segments show conserved divergence of groups

Direct sequencing of the 222 nt *rrn* intergenic spacer PCR products generated from avian *C. psittaci* strains 84/2334, Prk/Daruma, VS225, VS1, MNRh and MNOs provided unambiguous double-stranded sequences (respectively GenBank accession nos AJ310736, AF481048, AF481049,

Table 2. Primers and PCR conditions

Target	Primer	Sequence (5'→3')	Annealing temp. (°C)	Reference
<i>ompA</i>	ML-genoI	ATGAAAAAACTCTTGAAATCGG	55	This study
	ML-genoII	CCTTTATAGCCTCTTGTTTGTG		
<i>rrn</i> spacer	#16SF2	CCGCCCCGTACATCATGG	55	Everett <i>et al.</i> (1999)
	#23R	TACTAAGATGTTTCAGTTC		
<i>rnpB</i>	JB1	CGAACTAATCGGAAGAGTAAGGC	58	Herrmann <i>et al.</i> (2000a)
	JB2	GAGCGAGTAAGCCGG(A/G)TTCTGT		
Extrachromosomal plasmid	ML-PLF01	CAGAACGGTGTGTAGATT	52	This study
	ML-PLR01	AGGAGATAAGGTTGCTAA

Fig. 1. PCR and sequencing of extrachromosomal plasmid segment ORF8-ORF1. (a) Agarose gel separation. Lanes: 1, *C. psittaci* strain 6BC^T; 2, CE-LIF dsDNA 100 bp ladder (Bio-Rad); 3, *C. psittaci* strain 84/2334; 4, *C. abortus* strain B577^T. (b) Location of ORF8-ORF1 segment in *C. psittaci* plasmid.

AF481052, AF481051 and AF481050). Alignment of these data with orthologous *Chlamydiaceae* sequences showed that all were closely related to *C. psittaci* or *C. abortus*. Sequences for strains 84/2334 and Prk/Daruma were identical, although isolation, PCR and sequencing of these strains were carried out in laboratories located on separate continents. NJ analysis showed that strain 84/2334 is close to the base of the *C. psittaci* tree, sharing a common ancestor with *C. abortus*. Strains Prk/Daruma and 84/2334 differed from *C. abortus* strains by a single nucleotide polymorphism (SNP) at position 206. *C. abortus* was divergent from all *Chlamydiaceae* in this position (Fig. 2), although some of the branches were delineated from relatively few additional nucleotide differences. Strains GP1 NGP-17/11-14, GP3 NPG-LX 603C-12, M1 Bab-mouse-5 and R1 PNT-7 Montana rabbit have *rrn* intergenic spacer sequences identical to those of *C. abortus* strains EBA, OSP, A22 and B577^T. So, despite the diverse host spectrum, the *rrn* intergenic spacers of all sequenced *C. abortus* strains are identical. Divergence of the 84/2334 cluster from the Par1, 6BC^T and MN clusters was evident from direct examination of the spacer sequence data, NJ analysis (Fig. 2) and the 850 bases available for the 23S rRNA immediately downstream (data available in GenBank; analysis not shown). Relative to other *C. psittaci* strains, SNPs in strain 84/2334 at positions 186, 200 and 206 and a gap at position 194 were in a conserved portion of the spacer that is predicted to form a stem with a complementary downstream segment for 23S rRNA processing (Everett & Andersen, 1997). SNPs at positions 49, 50 and 90 were in hypervariable regions.

Multiple messages in MOMP

The reading frame of the cloned *C. psittaci* strain 84/2334 *ompA* gene was 1089 bp long. This segment encoded 363 deduced amino acids of the estimated 391 total amino acids

in the full-length MOMP. BLAST search analysis with MOMP indicated that strain 84/2334 was equally close to *C. psittaci* strains VS225, Par1, GD and CT1 and to *C. abortus* strains.

Fig. 2. NJ phylogenetic analysis of *rrn* intergenic spacers in *Chlamydophila* species. Numbers on branches indicate percentage bootstrap support. *Horizontal branches of *C. pecorum* and *C. pneumoniae* are shortened 10-fold. Sequences of abortion strains GP1 NGP-17/11-14, GP3 NPG-LX 603C-12, M1 Bab-mouse-5 and R1 PNT-7 Montana rabbit are identical to that of *C. abortus* strain EBA. Bar, 0.01 substitutions per site.

Amino acid differences between strain 84/2334 and these relatives were primarily in the four previously described hypervariable segments (VS1–VS4) (Baehr *et al.*, 1988). Examination of the five *ompA* conserved segments (CS1–CS5) of *C. psittaci* and *C. abortus* strains showed few deduced amino acid differences but large numbers of nucleotide differences (Table 3). These nucleotide differences revealed the probable order of *ompA* divergence in *Chlamydomophila*. NJ analysis of *ompA* CS sequences showed two *C. psittaci* lineages diverging: one contained the *C. psittaci* type strain 6BC^T and MN strains, while the other contained numerous *C. psittaci* strains (including 84/2334) and *C. abortus* (Fig. 3a). In the latter lineage, WC, NJ1 and TT3 diverged early; *C. psittaci* strains Par1, GD and CT1 clustered together, and the Par1 cluster, VS225, 84/2334, R54 and *C. abortus* strains each diverged later and separately (Fig. 3a). Strains GP1 NGP-17/11-14, GP3 NPG-LX 603C-12, M1 Bab-mouse-5 and R1 PNT-7 Montana rabbit have *ompA* sequences identical to that of *C. abortus* strain EBA. So, despite a wide range of hosts, *C. abortus* strains have almost identical *ompA* sequences. Bootstrap resampling provided support for this branching order. Sequences of *C. psittaci* strains (CT1, Par1, GD, VS225, R54 and 6BC^T) and of *C. abortus* strains (B577^T and LLG) showed nucleotide differences with reference to *C. psittaci* strain 84/2334 that were distributed unevenly among the CS sequences; just 1/10 of the SNPs encoded amino acid changes. None of these differences encoded functionally divergent amino

acids. Summing of the data in Table 3 provided a measure of this diversity: CS1 and CS5 had a total of 24 nucleotide differences that led to just five amino acid differences; CS2 and CS4 had 86 and 87 nucleotide differences, respectively, translating into a total of 11 amino acid differences. The 128 nucleotide differences in CS3 translated into 22 amino acid differences. The numbers of nucleotide and amino acid differences showed no correlation with insertion/deletion (indel) size or with apparent phylogeny (Table 3). All available *C. abortus ompA* sequences differed from the CS sequences of even the most closely related *C. psittaci* strains (including strain R54) by 16 unique point mutations.

In the four *ompA* VS sequences, no indels were required to align 84/2334 with VS225, Par1, GD or CT1, suggesting that these strains were closely related. One indel residue (3 nt) separated 84/2334 from strain R54, two from *C. abortus*, three from NJ1, five from WC and 11 from 6BC^T (Table 3). NJ analysis of the VS sequences was generally consistent with analysis of the CS sequences, except in the case of strain VS225 (Fig. 3a, b). Like CS analysis, NJ analysis of the VS sequences showed two major lineages. One lineage contained *C. psittaci* 6BC^T and MN strains, whereas the other contained *C. psittaci* strains CT1, Par1, GD, R54, VS225 and 84/2334, from which *C. abortus* diverged last. The VS sequences of strains WC, TT3 and NJ1 branched separately. Divergence of strain VS225, according to VS analysis, occurred just before the separation of *C. abortus*; according to CS analysis, however, strain VS225 separated several nodes closer to the base of the *C. psittaci* lineage. Strain 84/2334 shared eight VS point mutations with all members of the Par1/GD/CT1 cluster to the exclusion of all other strains; strain WC had four of the divergent Par-cluster bases (in VS2). Strain R54 had six point mutations (in VS1) that grouped it with the Par cluster; four mutations (VS3 and VS4) grouped R54 with *C. abortus* and were also present in 84/2334. *C. abortus* shared a set of 15 exclusive VS point mutations with 84/2334 and 11 of these with VS225. *C. abortus* VS sequences were uniquely distinguished from all *C. psittaci* strains by four point mutations.

The downstream non-coding segment between *ompA* and tRNA^{Gly} also revealed an order of divergence that was congruent with the CS divergence (data available in GenBank; analysis not shown).

Hypervariable segment diversity in *rnpB*

The PCR-amplified 388 bp portion of *rnpB* from *C. psittaci* strain 84/2334 was identical in sequence to that of strain R54 and differed by an SNP and a 2-base deletion from the sequence of *C. abortus* strain B577^T (Fig. 4). The 2-base deletion in 84/2334 was conserved among nine available *C. psittaci rnpB* sequences described by Herrmann *et al.* (2000a) (strains 6BC^T, GD, NJ1, WC, VS225, 360, N352, Cal10 and CP3). Strain 84/2334 *rnpB* differed from all nine *C. psittaci* sequences by 6 bases in four previously described *rnpB* hypervariable regions (P3, P12, P17 and P19; Fig. 4) (Herrmann *et al.*, 2000a).

Table 3. MOMP (*ompA*) sequence variation of *C. psittaci* strain 84/2334 compared with selected strains

CS variability is shown for nucleotides and amino acids. VS indels are shown for amino acid sequences. *C. abortus* strain LLG was described by Vretou *et al.* (1996). NK, Only a partial sequence was available.

Sequence characteristic	<i>C. psittaci</i>				<i>C. abortus</i>	
	CT1, GD, Par1	VS225	R54	6BC ^T	LLG	B577 ^T
VS indels	0	0	1	11	2	2
Nucleotide differences						
CS1	2	6	NK	5	1	1
CS2	16	14	9	25	11	11
CS3	18	22	17	29	21	21
CS4	10	11	5	16	22	23
CS5	2	1	0	2	2	2
Total	48	54	NK	77	57	56
Amino acid differences						
CS1	0	1	NK	1	0	0
CS2	2	0	0	2	0	0
CS3	4	5	3	2	4	4
CS4	0	1	0	1	2	3
CS5	2	0	0	1	0	0
Total	8	7	NK	7	6	7

Fig. 3. NJ phylogenetic analysis of *ompA* in *Chlamydia* species. (a) CS tree. *Horizontal branches of *C. pecorum* and *C. pneumoniae* strains are shortened 10-fold. Bar, 0.01 substitutions per site. (b) VS 50% bootstrap consensus tree. Numbers on branches indicate percentages bootstrap support. Sequences of abortion strains GP1 NGP-17/11-14, GP3 NPG-LX 603C-12, M1 Bab-mouse-5 and R1 PNT-7 Montana rabbit are identical to that of *C. abortus* strain EBA.

Fig. 4. *rnpB* sequence comparison. Sequences from *C. psittaci* strains 84/2334 and R54 are identical and contrast with those from *C. psittaci* 6BC^T and *C. abortus* B577^T. Gaps are indicated by dots; identity to *rnpB* of 84/2334 is indicated by dashes. Hypervariable regions P3, P12, P17 and P19 are indicated (Herrmann *et al.*, 2000a). Numbering is according to Brown (1998).

C. psittaci was named from infections caused by chlamydiae such as *C. psittaci* strain 84/2334 from psittacine birds. It is transmitted by inhalation, contact or ingestion among birds and to mammals. Psittacosis in birds and in humans often starts with flu-like symptoms and becomes a life-threatening pneumonia. Virulence among *C. psittaci* isolates varies; many strains remain quiescent in birds until activated under stress. Birds appear to be excellent vectors for the distribution of chlamydial infection because they feed on, and have access to, the detritus of infected animals of all sorts and are highly mobile (Everett *et al.*, 1999). In view of this, it is particularly intriguing that strain R54 was obtained in Antarctica from a brown skua. This bird breeds in the Southern Ocean from New Zealand to the Falklands and migrates to the Northern Hemisphere during the southern winter (Hermann *et al.*, 2000b). Thus, it is no surprise that isolates as closely related as Par1, GD and CT1 could be found in such diverse respective hosts and locations as a partridge in Britain, a duck in Germany and a turkey in California. Remarkably, only *C. psittaci* has been identified in birds, to date (Everett *et al.*, 1999). *C. psittaci* strains in birds infect mucosal epithelial cells and macrophages of the respiratory tract. Septicaemia eventually develops and the bacteria become localized in epithelial cells and macrophages of various organs (Vanrompay *et al.*, 1995).

humans (Table 1) (Rodolakis *et al.*, 1998; Everett *et al.*, 1999; Pospischil *et al.*, 2002). *C. abortus* has not been isolated from birds. *C. psittaci* and *C. abortus* were recognized as species primarily on the basis of differences in pathogenicity and DNA–DNA reassociation (Cox *et al.*, 1988; Fukushi & Hirai, 1989; Everett *et al.*, 1999).

In this report, we have presented evidence for the divergence of *C. abortus* from *C. psittaci*. Conservation in *rnpB*, *ompA* and *rrn* gene sequences of *C. psittaci* and *C. abortus* indicated that these species once shared a common ancestor. Divergence from this ancestor was evident in conserved segments of the *ompA* gene, in the *rrn* intergenic spacer and in additional segments. *C. psittaci* strains WC, NJ1 and TT3 had diverged the least from this common ancestor, while two *C. psittaci* lineages had undergone extensive divergence. One lineage contained 6BC^T and MN strains. The other lineage comprised progressively diverse strains, in which 84/2334, Prk/Daruma and R54 were intermediates in the evolution of *C. abortus* from *C. psittaci* and demonstrated characteristics of both species. The virtual absence of shared unique SNPs in conserved genetic loci of *C. psittaci* and *C. abortus* indicated that these strains did not diverge recently.

C. psittaci strain 84/2334 provides a link that was previously missing in our understanding of the divergence of *C. abortus* from the *C. psittaci* lineage. *C. psittaci* strain 84/2334 had DNA sequence identity with an extrachromosomal plasmid in duck strain N352 (pCpA1; Thomas *et al.*, 1997), with *rnpB* in strain R54 from a brown skua (originally characterized as *C. abortus*; Herrmann *et al.*, 2000b) and with the *rrn* intergenic spacer in parakeet strain Prk/Daruma (Fig. 2) (Fukushi *et al.*, 1987). These data confirmed the relatedness among these *C. psittaci* strains, while the accumulation of SNPs in *ompA* CS sequences suggested the antiquity of their separation (Table 3). Although *C. psittaci ompA* was heterogeneous and highly diverged, all available *C. abortus ompA* sequences differed from the CS sequences of even the

most closely related *C. psittaci* strains (including R54) by 16 SNPs. Total *ompA* CS nucleotide differences between *C. psittaci* strain 84/2334, *C. psittaci* and *C. abortus*, shown in Table 3 and in the NJ analysis (Fig. 3a), support the intermediate position of strains 84/2334, Prk/Daruma and R54 in the evolution of *C. abortus* from *C. psittaci*. The NJ tree of *ompA* CS sequences shows a strong resemblance to the quartet puzzling phylogenetic tree based on MOMP sequences described by Bush & Everett (2001).

Comparison of nucleotide sequences for the CS and VS sequences of *ompA* revealed the effects of selective pressures. *C. psittaci* strains VS225 and 84/2334 shared 11 unique VS point mutations with *C. abortus*, despite all other loci showing VS225 diverging early and 84/2334 as an intermediate (Fig. 3). Strain R54 shared only two of the 11 *C. abortus* VS mutations. This suggests that *C. psittaci* strain VS225 has evolved toward the *C. abortus*-like genotype, whereas strains R54 and 84/2334 have not. Such evidence is masked when comparisons look only at indels, amino acids, parsimony or NJ analysis of the entire *ompA*. Because the history of the evolution of these strains is not available to us, we cannot know what selective forces caused VS evolution in strain VS225 towards *C. abortus*. It has been proposed that random accumulation of nucleotide changes provides a fertile structural substrate of hypervariability (Chang & Casali, 1994). Accumulation of mutations in *C. psittaci ompA* has certainly provided a coding sequence prone to replacement mutations and amino acid substitutions. The discovery of convergent evolution in VS225 triggers important considerations, since VS regions are often relied upon for identifying chlamydiae through sequence analysis, RFLP (Vanrompay *et al.*, 1997) and mAb recognition. Reliance on such highly divergent sequences for classification can result in mistaken identification, as in the recent assignment of strain R54 to *C. abortus* (Herrmann *et al.*, 2000b). On the basis of our new understanding of these lineages, we hereby move R54 from *C. abortus* to *C. psittaci*.

Our findings of diversity and evolution in *C. psittaci* are consistent with reports based on 16S rRNA analysis (Pudjiatmoko *et al.*, 1997; Takahashi *et al.*, 1997; Pettersson *et al.*, 1997; Everett *et al.*, 1999; Bush & Everett, 2001). Pudjiatmoko *et al.* (1997) suggested that *C. psittaci* strain Prk/Daruma (D85710) was intermediate between *C. psittaci* and *C. abortus*, and Takahashi *et al.* (1997) had evidence for three intermediate isolates from parakeets (Prk 46, accession no. AB001809; Prk 48, accession no. AB001810; Prk 49, accession no. AB001811). CLUSTAL X analysis of these four strains shows that their 16S rRNA sequences are identical (data not shown) (Pudjiatmoko *et al.*, 1997; Takahashi *et al.*, 1997).

How does a bacterium that causes systemic disease in birds evolve into an agent of mammalian abortion? The answer will provide critical insights into the mechanisms of chlamydial virulence, and may eventually only be answered by genome sequence comparison. Until typing methods are

expanded, our ability to distinguish *C. psittaci* and *C. abortus* will continue to rely on ecological differences, mAbs and genetic data (16S or 23S rRNA signature sequences, *ompA*, cysteine-rich proteins, *kdtA* or *groEL*) (Everett & Andersen, 1997; Meijer *et al.*, 1997, 1999; Takahashi *et al.*, 1997; Wardrop *et al.*, 1999; Herrmann *et al.*, 2000a; Ochiai *et al.*, 2000; Bush & Everett, 2001). Because of this, we must utilize more than a single method and more than a single gene to characterize novel strains. Our study suggests that there is much to be learned about the separation of *C. psittaci* and *C. abortus*.

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