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Identification and characterisation of coding tandem repeat variants in *incA* gene of *Chlamydophila pecorum*

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Abstract – Bacteria of the family Chlamydiaceae are obligate intracellular pathogens of human and animals. *Chlamydophila pecorum* is associated with different pathological conditions in ruminants, swine and koala. To characterize a coding tandem repeat (CTR) identified at the 3' end of *incA* gene of *C. pecorum*, 51 strains of different chlamydial species were examined. The CTR were observed in 18 of 18 tested *C. pecorum* isolates including symptomatic and asymptomatic animals from diverse geographical origins. The CTR were also found in two strains of *C. abortus* respectively isolated from faeces from a healthy ewe and from a goat belonging to asymptomatic herds, but were absent in *C. abortus* strains isolated from clinical disease specimens, and in tested strains of *C. psittaci*, *C. caviae*, *C. felis* and *C. trachomatis*. The number of CTR repeats is variable and encode several motifs that are rich in alanine and proline. The CTR-derived variable structure of *incA*, which encode the Chlamydiaceae-specific type III secreted inclusion membrane protein, IncA, may be involved in the adaptation of *C. pecorum* to its environment by allowing it to persist in the host cell.

***Chlamydophila pecorum* / *incA* gene / coding tandem repeats**

1. INTRODUCTION

The family Chlamydiaceae is divided into two genera, *Chlamydia* and *Chlamydophila*. The *Chlamydia* genus includes three species: *C. trachomatis*, *C. muridarum* and *C. suis*. The *Chlamydophila* genus comprises six species: *C. psittaci*, *C. abortus*, *C. felis*, *C. caviae*, *C. pneumoniae* and *C. pecorum* [11]. *C. abortus* strains, efficiently colonise the placenta inducing abortion and weak neonates in ruminants [35]. *C. pecorum* strains have been isolated from ruminants, koalas and swine [24, 26]. These bacteria are associated with abortion, conjunctivitis, encephalomyelitis, enteritis, pneumonia and polyarthritis, but are commonly found in the intestines of animal without any clinical sign [36]. This species presents many genomic variations [9, 39].

Infection of host cells by all chlamydiae is mediated through a non-metabolically active form; the elementary body (EB), whereas intracellular multiplication progresses through the reticulate body (RB), a metabolically active, non-infectious form [28]. RB replication takes place entirely within an intracellular vacuole (the inclusion), whose membrane constitutes a barrier separating chlamydiae from the nutrient-rich cytoplasm. Modification of the inclusion membrane requires chlamydial protein synthesis, suggesting that the chlamydiae synthesize proteins engaged in interactions between the inclusion and cytosolic

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components [42]. To date, at least 11 distinct Inc proteins have been identified in the inclusion membrane of *C. trachomatis*-infected cells [4, 5, 43]. The first identified chlamydial Inc protein, termed IncA, involved a comparison of the serological response to *Chlamydia* in animals either infected with live organisms or immunized with killed chlamydial EB. Most Inc proteins have a unique bi-lobed hydrophobic domain of approximately 50–80 amino acids [34]. Microinjection studies [16] have provided direct evidence indicating that *C. trachomatis* IncA participates in the fusion of inclusions. In addition, inclusions containing mutant IncA do not fuse [47]. IncA also forms long fibers extending from the inclusion that are used as cytosolic tracks mediating the formation of secondary inclusions [48].

To date, coding tandem repeats (CTR) have not been described in chlamydial genomes including that of *Chlamydophila pecorum,* the subject of our study. Variable copy number tandem repeats have been observed in a number of prokaryotic genomes [1, 22]. The tandem repeats, which may be identical or partially degenerate, are usually classified among satellites: minisatellites (repeat units in the range 6–100 bp, spanning hundreds of base-pairs) and microsatellites (repeat units in the range 1–5 bp, spanning a few tens of nucleotides).

More recently, a number of studies have supported the notion that tandem repeats reminiscent of mini and microsatellites are useful as markers for the identification of pathogenic bacteria including those recently emerged from highly monomorphic species [2, 13, 19, 49]. Tandem repeats located within the promoter region of a gene may reveal an on/off switch of gene expression at the transcriptional level [50, 51]. CTR with a repeat unit length a multiple of three are contained within and contribute to a coding sequence, such that variations in the number of copies modify the gene product itself [53]. Tandem repeats within coding regions whose repeat unit length is not a multiple of three can induce a reversible premature end of translation when a mutation changes the number of repeats [17].

In this study, we describe, a coding tandem repeat identified in the *incA* gene via screening a genomic library of *C. pecorum*. The CTR is characterized and its existence in a large number of chlamydial strains belonging to several species is documented.

2. MATERIALS AND METHODS

2.1. Chlamydia isolates

Fifty one strains representing six species of the family Chlamydiaceae were used in this study: 19 strains of *C. pecorum*, 21 of *C. abortus*, 8 of *C. psittaci*, 1 of *C. caviae*, 1 of *C. felis* and 2 of *C. trachomatis* (Tab. I). All strains were propagated in the yolk sac of chicken embryos and stored at –70 °C as previously described [35]. For library construction, EB of *C. pecorum* M14 were purified according to [8].

2.2. Genomic library construction

Genomic DNA was extracted from purified EB as described [7] of *C. pecorum* M14 (isolated from an aborted goat in Morocco), restriction digested with *Sau*3A (Promega, Charbonnieres, France) enzyme and fragments (0.1–12 kb) in length were use to generate a lambda ZAP library (ZAP Express Predigested Vector Kit, Stratagene, Massy, France). The library was screened with anti-*C. pecorum* M14-specific serum from infected sheep (experimentation).

2.3. PCR

Genomic DNA was extracted from 15–50 µL of yolk sac fluid using the tissue protocol of the DNeasy Tissue Kit (QIAGEN S.A., Courtaboeuf, France). PCR was performed according to the GoTaq Flexi DNA Polymerase (Promega) protocol. The primers used to generate a fragment (454 bp) which contained the tandem repeats were B15-F (5′ CAAGAACAGTTGCGTCCTG) and IncA-S-R (5′ GTGTGAGATGGCTCTTTATG). PCR (final volume, 25 µL) consisted of DNA denaturation at 94 °C for 5 min followed by 30 cycles of amplification with Thermocycler (Biometra, Goettingen, Germany). Each cycle consisted of a denaturation step at 94 °C for 30 s, an annealing step at 63 °C for 45 s, an extension step at 72 °C for 1.5 min, and a chain elongation at 72 °C for 10 min. Finally, 10 µL

Table I. Strains used in this study.

Strain	Geographic origin	Host	Pathology	Passage history (number)	Reference
C. pecorum					
M14	Morocco	Goat	Abortion	6	[32]
AKT	Tunis	Sheep	Abortion	2	[a]
AB10	France	Sheep	Abortion	5	[30]
VB2	France	Sheep	Orchitis	1	[a]
LW679	USA	Sheep	Arthritis	2*	[30]
SBE	England	Cattle	Encephalomylitis	3*	[b]
E58	USA	Cattle	Encephalomylitis	unknown	[27]
824	Scotland	Sheep	Conjunctivitis	2*	[25]
BE53	England	Cattle	Healthy (Feaces)	2*	[b]
iB1, iB2, iB3, iB4 and iB5	France	Sheep	Healthy (Feaces)	5, 3, 4, 3, 4	[40]
R69 and W73	Ireland	Sheep	Healthy (Feaces)	3*, 2*	[40]
iC2, iC3 and iC4	France	Goat	Healthy (Feaces)	3, 3, 5	[40]
C. abortus					
AB7	France	Sheep	Abortion	6*	[12]
H574	Scotland	Sheep	Abortion	4*	[25]
S26/3	Scotland	Sheep	Abortion	4*	[25]
AB1, AB2, AB13 and AB15	France	Sheep	Abortion	6, 4, 5, 2	[36]
AB16	France	Sheep	Abortion	3*	[6]
LLG and POS	Greece	Sheep	Abortion	3*, 3*	[45]
AV1	France	Cattle	Abortion	6	[9]
BA1	England	Cattle	Abortion	1*	[15]
AC1	France	Goat	Abortion	4	[9]
1H and 1B	France	Sheep	AB7 Mutant	2, 3	[38]
109–75	France	Sheep	Pneumonitis	2*	[9]
VB1	France	Sheep	Orchitis	1	[9]
OC1	France	Sheep	Conjunctivitis	6	[9]
1H/77	Scotland	Sheep	Arthritis	2*	[25]
MO907	USA	Sheep	Healthy (Feaces)	4*	[46]
iC1	France	Goat	Healthy (Feaces)	4	[9]
C. psittaci					
VS1	USA	Parrot	Psittacosis	1*	[3]
L2A	France	Bird	Ornithosis	1*	[40]
GR9	USA	Duck	Ornithosis	1*	[c]
NJ1	USA	Turkey	Ornithosis	1*	[3]
VS225	USA	Psittacine	Psittacosis	1*	[c]
9105983	France	Dinghy	Psittacosis	1	[10]
940673	France	Calopsitte	Psittacosis	1	[10]
9106568	France	Dove	Ornithosis	1	[10]
C. caviae					
GPIC	USA	Guinea- Pig	Conjunctivitis	2*	[44]

Table I. Continued.

Strain	Geographic origin	Host	Pathology	Passage history (number)	Reference
C. felis					
FePn	USA	Cat	Live vaccine	1*	[40]
C. trachomatis					
Serovar D strain D	Greece	Human	Conjunctivitis	2*	[d]
Serovar E strain E	Greece	Human	Conjunctivitis	1*	[d]

[a] Isolated at INRA, UR1282, Infectiologie Animale et Santé Publique, Centre de recherche de Tours, France.
[b] Isolated by M. Dawson, Virology Department, Central Veterinary Laboratory, Weibridge, UK.
[c] Isolated by A. Andersen, National Animal Disease Center, USDA, USA.
[d] From E. Vretou, Institute Pasteur Hellenique, Athens, Greece.
* Number of passages performed at INRA after receiving the strain.

of the PCR product was analyzed by 1.5% agarose gel electrophoresis and ethidium bromide stain.

2.4. *incA* gene sequencing

The nucleotide sequence of *incA* from *C. pecorum* strains and strains of *C. abortus* that tested positive by PCR, was determined by automated sequencing (Genome Express, Meylan, France). Primers used to amplify the 1.3 kb fragment including *incA*, were IncA-S-F: (3'TATCGTAATACCAAACCACT) and IncA-S-R: (3'GTGTGAGATGGCTCTTTATG) according to the GoTaq Flexi DNA Polymerase (Promega) protocol with annealing temperature of 52 °C and other PCR conditions as reported above. Amplified DNA was purified with the QIAquick PCR Purification Kit (QIAGEN S.A.). The primer pairs used in this study were derived from the genome sequence of *C. pecorum* E58 obtained by Myers and Bavoil (Genbank accession numbers are provided in Tab. II). Multiple alignments were performed using CLUSTAL W (1.83)[1] and the *C. pecorum* E58 sequence as reference.

3. RESULTS

3.1. Identification of a CTR in *incA*

The screening of the lambda ZAP library led to the isolation of a clone carrying a 1363 bp DNA fragment encoding an antigen of 35 kDa. Sequence analysis of this clone in pBK-CMV revealed two open reading frames (ORF). Comparison with the genome of *Chlamydophila pecorum* E58 revealed that ORF1 is 97% identical with a segment of *incA* of *Chlamydophila pecorum* E58 and only weak homology (< 25%) with other published chamydial genomic sequence[2]. Subsequent sequence analysis revealed that ORF1 of *C. pecorum* M14 contained CTR of nine nucleotides repeated 22 times. PCR amplification of the corresponding *incA* segment was then attempted from 51 strains from different species of the Chlamydiaceae (Tab. I) and the amplicons submitted to sequence analysis. Although an *incA* fragment of variable size (Fig. 1), was amplified from all tested *C. pecorum* strains and two *C. abortus* strains, (MO907 and iC1) isolated from the faeces of healthy animals, PCR amplification was negative for *C. abortus* strains isolated from clinical disease specimens, and for tested strains of *C. psittaci*, *C. caviae*, *C. felis* and *C. trachomatis*.

3.2. Characterization of the *incA* CTR

Several types of repeat motifs were observed in the 3' segment of *incA*. All

[1] Available online: http://www.ebi.ac.uk/clustalw/ [consulted 08/06/2007].

[2] Basic Local Alignment Search Tool [online]: http://ncbi.nlm.nih.gov/blast/ [consulted 19/07/2007].

Table II. Summary table of the CTR motifs in *incA* sequences[a].

Strain	Gene bp	Protein aa	Motif 1 APA	Motif 2 APAPE	Motif 3 APEVPA	Motif 4 APE	Accession No.
M14	1071	356	22				EU340814
VB2	1008	335	15				EU340824
iB3	999	332	14				EU340827
SBE	981	326	12				EU340823
E58	981	326	12				EU837066
iB5	984	327	12				EU340817
LW679	969	322	11				EU340821
AB10	954	317	9				EU340815
MO907*	954	317	9				EU340812
iC1*	954	317	9				EU340813
AKT	945	314	8				EU340816
BE53	945	314	8				EU340808
iB4	1023	340	10	4			EU340826
iC2	1098	365	6	12			EU340818
iC3	1083	360	6	11			EU340820
iC4	1098	365	6	12			EU340819
R69	1005	334	2	8			EU340822
W73	1005	334	2	8			EU340825
824	1053	350	4		8		EU340809
iB2	1008	335	5		3	4	EU340810
iB1	999	332	5		3	3	EU340811

[a] Gene and predicted protein size, number of the repeats for every motif and Genbank accession numbers are shown for 19 *C. pecorum* strains and 2 *C. abortus* strains (*).

sequenced strains presented a motif of nine nucleotides, GCTCCAGCG, (motif 1) encoding amino acids APA with 3 to 22 repetitions (Tab. II). A second motif of 15 nucleotides, GCTCCAGCTCCAGAG, encoding APAPE (motif 2) was present in six strains with 4–12 repetitions. A third motif of 18 nucleotides, GCTCCAGAGGTTCCAGCG, encoding APEVPA (motif 3) was found in three strains with three to eight repetitions. A fourth motif present in two strains was composed of nine nucleotides, GCTCCAGAG encoding APE (motif 4), which was repeated three or four times. These motifs allowed to classify the strains into three groups.

A first group of 11 strains harbouring motif 1 only, includes 10 *C. pecorum* strains (7 associated with different diseases and 3 isolated from faeces) and 2 *C. abortus* strains (Fig. 2). A second group includes seven *C. pecorum* strains presenting 2 motifs: 5 strains isolated from faeces containing motifs 1 and 2 in tandem (Fig. 3). Two variations on the theme were *C. pecorum* IB4, in which motif 2 is enclosed by two motifs 1 (Fig. 4), and 824 isolated from conjunctivitis which has motifs 1 and 3 (Fig. 5). A third groups included 2 intestinal *C. pecorum* strains iB1 and iB2 presenting three motifs (motif 1, 3 and 4) (Fig. 6).

4. DISCUSSION

The coding tandem repeat found in *incA* of all tested *C. pecorum* strains and two *C. abortus* strains isolated from the faeces of healthy animals was neither present in other *C. abortus* strains isolated from clinical disease specimens, nor from tested *C. psittaci*, *C. caviae*, *C. felis* and *C. trachomatis* strains. Seven out of eight *C. pecorum* strains isolated from animals with clinical symptoms including abortion (three strains) were classified in a single group (group 1). Although this grouping provides hints of a

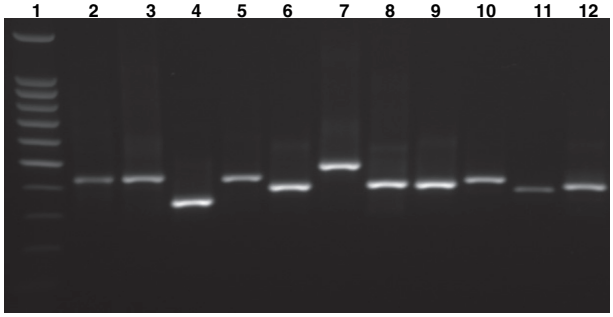

Figure 1. Amplification of *incA* sequence containing the coding tandem repeat (300-500 bp) on DNA extracts of *C. pecorum* strains. Lane 1: 100 bp ladder; Lanes 2-12: *C. pecorum* M14, 824, AB10, LW679, R69, iC2, VB2, W73, iB4, BE53, and iB3.

possible association with disease, additional *C. pecorum* strains from symptomatic or asymptomatic animals would need to be examined to establish an association of the motifs with virulence.

The motifs of IncA are rich in the amino acids alanine and proline and it is striking that many proline-rich sequences are repeated in proteins, e.g. (xP) or (xPy). Some of these sequences have unknown functions, but many of them appear to be involved in processes that increase bacterial virulence. Indeed, proteins with proline-rich CTR are often enhanced in their ability to interact with other proteins or to cross cell membranes.

Mutants of *Listeria monocytogenes* lacking proline-rich repeats domain of the ActA protein, a key factor in bacterium-host cell microfilaments interactions, moved two times slower than the wild type [21]. Attachment of *Mycobacterium avium* to the extracellular matrix protein fibronectin, a key to the virulence of several extracellular pathogenic bacteria binding to mucosal surfaces, is mediated by a fibronectin-attachment protein which contains CTR of AP [41]. However as the Chlamydiaceae are not free in the cytosol and as the IncA protein is not present on the extracellular form of chlamydiae, but only expressed after attachment to the host-cell [33], a similar role in attachment or intracellular mobility is not directly applicable to IncA CTR of *C. pecorum.*

The procyclin protein of *T. brucei* contains a long DP or EP repeat enabling the protein to extend out from the cell membranes [52]. IncA is likewise translocated across the membrane of the inclusion to facilitate interaction with cytoplasmic components of the host cell [33]. However IncA translocation to the outer face of the inclusion membrane also occurs in *Chlamydia* species with IncA proteins lacking the AP repeats.

In other examples, the variation in copy number within coding tandem repeats has been shown to alter the ability of antibodies to bind to bacterial antigens: addition of tandem repeats in the PAc protein of *Streptococcus mutans*, which normally contains three long repeated regions, induces higher antibody production than the native peptide [18]. The IncA CTR present at the C-terminal end of the protein could therefore affect the conformation of the protein inducing changes in its antigenicity, as previously observed. Size variation in surface proteins Lmp1 and Lmp3 of *Mycoplasma hominis* has also been correlated to tandem repeats at the C-terminal end of the proteins [20].

All examples described above illustrate the importance of the CTR in enhancing specific properties of bacterial virulence factors. It is surprising therefore that CTR are observed in *C. pecorum* which is regarded as the least virulent species of Chlamydiaceae. However we note that 7 out of 8 strains isolated from symptomatic disease samples belong to a single group that has only one motif (APA) with a different number of repeats. In addition, the two encephalomyelitis *C. pecorum*

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M14  272 QQAAAPAAPAAPAAPAAPAAPAAPAAPAAPAAPAAPAAPAAPAAPAAPAAPAAPAAPAAPAAPAAPAAPAAPAAPENN 346
VB2      QQAAAPAAPAAPAAPAAPAAPAAPAAPAAPAAPAAPAAPAAPAAPADPA--------------------PENN 325
IB3      QQAAAPAAPAAPAAPAAPAAPAAPAAPAAPAAPAAPAAPAAPAAPD----------------------PENN 322
SBE      QQAAAPAAPAAPAAPAAPAAPAAPAAPAAPAAPAAPAAPAAPA-----------------------------PENN 316
E58      QQAAAPAAPAAPAAPAAPAAPAAPAAPAAPAAPAAPAAPAAPA-----------------------------PENN 316
IB5      QQAAAPAAPAAPAAPAAPAAPAAPAAPAAPAAPAAPAAPAAPA-----------------------------PENN 317
LW679    QQAAAPAAPAAPAAPAAPAAPAAPAAPAAPAAPAAPAAPD--------------------------------PENN 312
IC1      QQAAAPAAPAAPAAPAAPAAPAAPAAPAAPA-----------------------------------------PENN 307
MO907    QQAAAPAAPAAPAAPAAPAAPAAPAAPAAPA-----------------------------------------PENN 307
AB10     QQAAAPAAPAAPAAPAAPAAPAAPAAPAAPA-----------------------------------------PENN 307
AKT      QQAAAPAAPAAPAAPAAPAAPAAPAAPA--------------------------------------------PENN 304
BE53     VHAAAPAAPAAPAAPAAPAAPAAPAAPEN-------------------------------------------NDNN 304
         ..************************                                          ..**
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Figure 2. Alignment of predicted amino acid sequences of the *incA* CTR region in 10 *C. pecorum* strains and 2 *C. abortus* intestinal strains.

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IC2 272 QQAAAPAAPAAPAAPAPAAPAAPAPEAPAPEAPAPEAPAPEAPAPEAPAPEAPAPEAPAPEAPAPEAPAPEAPAPEAPAPEAPAPENN 355
IC4     QQAAAPAAPAAPAAPAPAAPAAPAPEAPAPEAPAPEAPAPEAPAPEAPAPEAPAPEAPAPEAPAPEAPAPEAPAPEAPAPEAPAPENN 355
IC3     QQAAAPAAPAAPAAPAPAAPAAPAPEAPAPEAPAPEAPAPEAPAPEAPAPEAPAPEAPAPEAPAPEAPAPEAPAPEAPAPE-----NN 350
W73     QQAAAPAAPA-----------APAPEAPAPEAPAPEAPAPEAPAPEAPAPEAPAPEAPAPE--------------------NN 324
R69     QQAAAPAAPA-----------APAPEAPAPEAPAPEAPAPEAPAPEAPAPEAPAPEAPAPE--------------------NN 324
        **********           ****************************************                      **
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Figure 3. Alignment of predicted amino acid sequences of the *incA* CTR region in five *C. pecorum* intestinal strains.

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IB4 272 QQAAAPAAPAVPAAPAPEAPAPEAPAPEAPAPEAPAAPAAPAAPAAPAAPAAPDPENNDNNNDDNAAS 340
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Figure 4. Alignment of predicted amino acid sequences of the coding tandem repeat of the *incA* gene of *C. pecorum* IB4.

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824  272  QQAAAPAAPAAPAAPEVPAAPEVPAAPEVPAAPEVPAAPEVPAAPEVPAAPEVPAASEVSAAPDPENN 340
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Figure 5. Alignment of predicted amino acid sequences of the *incA* CTR region in *C. pecorum* conjunctivitis strain 824.

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IB2  272 QQAAAPAAPAVPAAPAAPAVPEAPEVPAAPEVPAAPEAPEAPEAPEAPAPENNDNNNDDNAAS 335
IB1      QQAAAPAAPAVPAAPAAPAVPEAPEVPAAPEVPAAPEAPEAPE---APAPENNDINNDDNAAS 332
         ****************************************   ******** ********
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Figure 6. Alignment of predicted amino acid sequences of the *incA* CTR region in two *C. pecorum* intestinal strains.

(E58 and SBE) strains had the same number of repeats in spite of their different geographical origins. The same high level of homology was found for the two *C. abortus* strains (isolated from healthy animals) and abortive *C. pecorum* strains AB10 and AKT isolated in France and Tunisia respectively. The high sequence similarity of *incA* between the three intestinal *C. pecorum* isolates iC2, iC3 and iC4 (which were isolated from three animals belonging to the same herd) added to the previously observed similarity by typing with monoclonal antibodies by indirect microimmunofluorescence (MIF) [40], leading us to postulate that these are three different isolates of the same original strain. Passages in eggs or in the animal host may have played a role in the observed minor differences (± 1 repetition of CTR) as for the intestinal ovine *C. pecorum* iB1 and iB2 isolates.

CTR may play a role in the adaptation of chlamydiae to their environment. Variability in the number of repeats in various cell-surface genes of *Candida albicans* clinical isolates may allow rapid adaptation to a fluctuating environment and/or immune evasion [54, 55]. *C. pecorum* is often found in the intestines of healthy animals and it is able to adapt to and persist in the host cell without destroying it, owing to its slow growth rate [23, 29, 37]. Persistence also correlates to the non-fusogenic inclusions phenotype as predicted by biomathematic modelling [31] and confirmed by observation: indeed, non fusogenic strains of *C. trachomatis* are more frequently associated with sub-clinical infection than their fusogenic counterparts [14]. To further characterize the impact of the CTR on the structure/function of the inclusion in various strains of *C. pecorum*, future studies of in vivo IncA expression may allow to establish the full range of antigenic variability of this protein among different strains.

In conclusion, the function of the IncA AP-rich CTR variants of *C. pecorum*, a species which is less virulent than other *Chlamydophila* species such as *C. psittaci* and *C. abortus*, is unknown. However, it is tempting to speculate that it may be involved in the adaptation of the organism to its environment and/or for immune evasion thereby allowing *C. pecorum* to persist in the host cell for extended periods of time.

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