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Effect of long-term laboratory propagation on *Chlamydia trachomatis* genome dynamics

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ABSTRACT

It is assumed that bacterial strains maintained in the laboratory for long time shape their genome in a different fashion from the nature-circulating strains. Here, we analyzed the impact of long-term *in vitro* propagation on the genome of the obligate intracellular pathogen *Chlamydia trachomatis*. We fully-sequenced the genome of a historical prototype strain (L2/434/Bu) and a clinical isolate (E/CS88), before and after one-year of serial *in vitro* passaging (up to 3500 bacterial generations). We observed a slow adaptation of *C. trachomatis* to the *in vitro* environment, which was essentially governed by four mutations for L2/434/Bu and solely one mutation for E/CS88, corresponding to estimated mutation rates from 3.84×10^{-10} to 1.10×10^{-9} mutations *per* base pair *per* generation. In a speculative basis, the mutations likely conferred selective advantage as: (i) mathematical modeling showed that selective advantage is mandatory for frequency increase of a mutated clone; (ii) transversions and non-synonymous mutations were overrepresented; (iii) two non-synonymous mutations affected the genes CTL0084 and CTL0610, encoding a putative transferase and a protein likely implicated in transcription regulation respectively, which are families known to be highly prone to undergone laboratory-derived advantageous mutations in other bacteria; and (iv) the mutation for E/CS88 is located likely in the regulatory region of a virulence gene (CT115/*incD*) believed to play a role in subverting the host cell machinery. Nevertheless, we found no significant differences in the growth rate, plasmid load, and attachment/entry rate, between strains before and after their long-term laboratory propagation. Of note, from the mixture of clones in E/CS88 initial population, an inactivating mutation in the virulence gene CT135 evolved to 100% prevalence, unequivocally indicating that this gene is superfluous for *C. trachomatis* survival *in vitro*. Globally, *C. trachomatis* revealed a slow *in vitro* adaptation that only modestly modifies the *in vivo*-derived genomic evolutionary landscape.

1. Introduction

Random mutation events create variation that is purged by selection in the never-ending process of microbial evolutionary adaptation. The specific causes of variation are hardly traceable as they mostly depend on fluctuations in the environment, which may be as diverse as immune pressure, lack of nutrients, or changes in physiological conditions, such as pH or temperature. In the bacteria research field, it is important to understand the association between the replication associated errors, the frequency of selective sweeps that purge those errors, and the ultimate impact of these dynamics on organism adaptation to new environments. For DNA-based microbes, reported mutation rates *per* base pair *per* generation vary up to four orders of magnitude, and high mutation rates have been found in laboratory asexual bacterial populations (Conrad et al., 2011; Sniegowski et al., 2000).

Several studies have evaluated the microbe genomic alterations due to laboratory passaging but limited data are available so far for obligate intracellular bacteria (Labiran et al., 2012; Stothard et al., 1998), where the interaction with the host cell line governs the evolutionary process. Indeed, according to the Red Queen Hypothesis, interacting species undergo an arms race of continuous adaptation and counter-adaptation that drives molecular evolution (Van Valen, 1973). By extrapolating this scenario to the *in vitro* system, this constitutes a rational basis for assuming that bacterial strains "adapted" to the laboratory for a long time shape their genome in a different fashion from the wild-type strains. Indeed, the latter deal with different biological host niches encompassing dissimilar immunological and physiological status, as well as competing microbiota. This is

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reflected by the bacterium *Chlamydia trachomatis*, a human pathogen that causes non-invasive (restricted to the mucosal epithelium of ocular or genital tract) or invasive (mainly through the dissemination to lymph nodes leading to an inguinal lymphadenopathy named lymphogranuloma venereum – LGV) diseases. This obligate intracellular pathogen has a unique infectious cycle of about 48 h (Lambden et al., 2006), and alternates between an infectious form (elementary body – EB) and a replicative form (reticulate body – RB) that multiplies within a host vacuole named inclusion. The culture isolation of *C. trachomatis* dates back to the 1950s (Tang et al., 1957) allowing the isolation of "prototype" strains, which were collected decades ago and have been laboratory maintained since then. These historical isolates have been focused by thousands of studies as they are accessible to laboratories worldwide, allowing cross-comparison and complementation of results. However, the genomic stability of these strains has been questioned as there is the assumption that the laboratory passaging has been affecting their genomic make-up (Gomes, 2012).

It was our goal to evaluate the impact of laboratory propagation on *C. trachomatis* genome dynamics. We performed a long-term serial *in vitro* expansion of both a prototype strain with a long history of laboratory passaging and a clinical isolate collected from a woman with pelvic inflammatory disease (that has not been *in vitro* passaged) during over a year (up to 3500 bacterial generations), and compared the full-genome sequences before and after the strains' propagation.

2. Material and methods

2.1. Long-term C. trachomatis propagation in tissue culture

The present study involved the long-term *in vitro* propagation of two *C. trachomatis* strains in the generally used HeLa229 cell line: the historical prototype strain L2/434/Bu (invasive genital strain/serovar L2) and the clinical isolate E/CS88 (non invasive genital strain/serovar E). L2/434/Bu was isolated in 1968 in California (USA) from an inguinal bubo (Schachter and Meyer, 1969). This strain was initially accessioned as an egg passaged preparation, and subsequently maintained in human cell lines at ATCC since then. We opted to use this prototype strain both because this isolate has been one of the most studied *C. trachomatis* strains worldwide, and to test if an evolutionary different scenario would be obtained when compared with a clinical isolate that had never been laboratory propagated before. In our study we used L2/434/Bu strain obtained from the ATCC collection. The strain E/CS88 belongs to the culture collection of the Portuguese National Institute of Health, and was isolated in 1993 from a woman with chlamydial pelvic inflammatory disease. The diagnostic was performed by COBAS Amplicor PCR (Roche Molecular Systems, Branchburg, NJ, USA) and culture, as previously described (Catry et al., 1995), and both the original endocervical swab and the harvested infected HeLa229 cells were stored in liquid nitrogen since then.

The initial inoculation of strains was generally performed using previously described techniques (Borges et al., 2010). Briefly, each strain was inoculated onto confluent *Mycoplasma*-free HeLa229 cell monolayers (in T25 cm^2 flasks) by centrifuging for 1 h at 34 °C at 2200 rpm. Subsequently, the cultures were incubated for 1 h at 37 °C, 5% CO_2, the cell medium was replaced by an enriched medium (MEM 10% fetal bovine serum, vitamins, non-essential aminoacids, glucose and 0.5 μg/ml cycloheximide), and cultures were allowed to grow at 37 °C, 5% CO_2. The inclusion development was regularly monitored through phase-contrast microscopy by checking the inclusion size in order to precisely decide when the passage is needed. In a continuous fashion, the bacterial-saturated cells were harvested by trypsinization and transferred to new T25 cm^2 flasks containing a suspension of fresh HeLa229 cells (which enable the still-dividing RBs to proceed with the developmental cycle). When necessary, and specifically in order to avoid the culture decline, the suspension of infected-cells was sonicated (Vibra Cell, Bioblock Scientific) for disrupting eukaryotic cells and bacterial releasing. Subsequently, the cell debris was discarded through low-speed centrifugation, and the *Chlamydia*-enriched supernatant was then inoculated by centrifugation (as described above). As our goal was to study the mutations generated during the replicative process in the laboratory propagation, we adopted trypsinization as the major propagation method because sonication discards dividing bacteria. The regular microscopy visualization allowed us to make sure that the inclusions continued their regular development after trypsinization, although we cannot exclude that some inclusions may be lost. Both L2/434/Bu and E/CS88 cultures were serially maintained for over one year, which constituted about 250 tissue culture passages. Considering the use of different harvesting methods as well as the fluctuations on growth rate observed for both strains during the long-term experiment, the passages were not performed at rigid time intervals (frequently, passages were spaced less than 36 h).

2.2. Preparation of DNA for sequencing

For reading simplification and when the need arises, an "(i)" or "(f)" will be added to the designation of the strains. Thus, L2/434/Bu(i) and E/CS88(i) represent the strains in the initial stage of the evolution experiment, whereas L2/434/Bu(f) and E/CS88(f) refers to the final stage. For each *C. trachomatis* strain, we performed a scale-up from both initial and end-point cultures in order to generate sufficient quantities of DNA for full-genome sequencing. *Chlamydia*-enriched cells in up to 10 T75 cm^2 flasks were harvested by using glass beads, re-suspended with cold phosphate-buffered saline (PBS) and subjected to a discontinuous density gradient purification procedure adapted from previous studies (Caldwell et al., 1981; Lefebvre and Orfila, 1980) to obtain chlamydial material free of host-cell contaminants. Briefly, cell suspensions were pooled, ruptured by sonication and centrifuged at 500*g* for 10 min at 4 °C. The resulting supernatant was further centrifuged at 30000*g* for 1 h at 4 °C. The final pellet was re-suspended in PBS, homogenized by sonication, and finally pipetted over layers of discontinuous urographin [urographin 76%: sodium amidotrizoate (0.1 g) and meglumine amidotrizoate (0.66 g); Bayer – Portugal)] gradients: 3 ml of 60%, 10 ml of 52%, 10 ml of 45% and 10 ml of 30% urographin (vol/vol) in 40 ml tubes. These gradients were centrifuged at 26500*g* for 1.5 h. The EB fraction (located at the 45/52% urographin interface) and the RB fraction (located at the 30/45% urographin interface) were collected, diluted in PBS, and then centrifuged at 30000*g* for 1 h at 4 °C. The resulting pellets were washed with PBS to remove residual urographin, and re-suspended in PBS. DNA was extracted using the DNA Mini Kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. Prior to sequencing, several assays were performed to assess the purity and quality of DNA recovered by this method. We preliminary checked the degree of human DNA contamination in both EB and RB fractions by quantifying the copy number of *C. trachomatis per* copy number of HeLa229 genomes using a previously described real-time quantitative PCR (Gomes et al., 2006). We opted to use exclusively the EB fractions as we found a degree of contamination with host-cell DNA of at least 70-fold lower in the fraction of EBs (less than 0.01% of contaminant host-cell DNA copies in each EB sample). The quality of DNA was verified by agarose gel electrophoresis, and A260/A280 readings yielded optimal results of ~1.8. Finally, the DNA was quantified by fluorometry (Quant-iT Picogreen, Invitrogen), and ~500 ng of highly pure DNA from L2/434/Bu(i), L2/434/Bu(f), E/CS88(i) and E/CS88(f) were used for full-genome sequencing.

2.3. DNA sequencing and assembly

The chromosome and plasmid of both L2/434/Bu and E/CS88 were sequenced before and after their intensive continuous laboratory passaging by using a Roche 454 GS FLX Titanium according to the standard manufacturer's instructions (Roche-454 Life Sciences, Branford, CT, USA) at Biocant (Cantanhede, Portugal). A mean of 163,591 reads (after removing vestigial reads resulting from contamination with human and *Mycoplasma* DNA) with an average length of 443 bp were generated for the four samples, representing a coverage between 56- and 75-fold (Supplementary Table 1). The reads were mapped through the GS Reference Mapper v2.6 software, using as reference the available sequences of previously full-sequenced *C. trachomatis* strains. For the analysis of L2/434/Bu, as this strain had already been sequenced in 2008 (Thomson et al., 2008), the available genome and plasmid sequences (GenBank accession number NC_010287 and NC_010285, respectively) were used. For the E/CS88 isolate, we used the available chromosome sequence from the strain E/Bour (GenBank accession number HE601870.1) and the plasmid sequence of the strain E/SotonE4 (GenBank accession number HE603232.1). Given the non-repetitive nature of the *C. trachomatis* genome, a low number of contigs (no more than five) were obtained for each sample, where the plasmid sequence was presented as a single contig. Globally, the contigs covered more than 99.4% and 100% of to the length of the chromosome and plasmid sequences of the references, respectively (Supplementary Table 1). All variant positions between the samples and the references revealed by the GS Mapper software were carefully inspected. Only unambiguous base variants [i.e., noticed by both forward and reverse readings in a similar percentage of reads and not laying in the 454/Roche error-prone long homopolymeric regions (Thomson et al., 2008)] were considered. Finally, the chromosomal consensus contigs were simply joined taking into account the sequence of the contig boundaries and the genome of the reference sequences. The sequences were submitted to GenBank database (http://www.ncbi.nlm.nih.gov/GenBank/index.html), and are available under accession numbers CP003963-CP003966 (for L2/434/Bu) and CP003970-CP003971 (for E/CS88 plasmid). For E/CS88 chromosome, whole genome shotgun projects have been deposited under the accession ANOM00000000-ANON00000000. The versions described in this paper are the first versions, ANOM01000000-ANON01000000.

2.4. Identification of mutations due to laboratory passaging

The mutations detected between sequences retrieved from the initial and final stages were classified according to their frequency in the bacterial population. Thus, when base variants were supported by all sequence reads, they were considered as fixed mutations (i.e., carried by all clones in the population). On the other hand, as the high achieved coverage (56- to 75-fold) allowed the identification with great confidence of nucleotide positions revealing mixture of clones, the corresponding mutations were classified as: (i) high-frequent (more than 50% of reads support the mutation, i.e., carried by the majority of the clones); or (ii) low-frequent (less than 50% of the reads carry this nucleotide change, i.e., carried by low-frequent clones that have risen to a measurable frequency). For each identified mutation, we further assessed its chromosome location, structural class (transversions or transitions) and its effect at amino acid level (synonymous or non-synonymous mutations) based on fully-sequenced *C. trachomatis* strains for which complete annotation was performed (Carlson et al., 2005; Stephens et al., 1998; Thomson et al., 2008).

2.5. Impact of laboratory propagation on C. trachomatis fitness

We evaluated differences in *C. trachomatis* fitness before and after laboratory propagation by evaluating the growth rate, plasmid load *per* strain and attachment/entry rate. All these assays were performed in duplicate. The growth kinetic of the strains under evaluation was assessed through the calculation of the bacterial generation time (doubling time), which also allowed the extrapolation of the total number of bacterial generations reached during the ~1 year experiment. For this, we collected samples from four stages of the experiment including the initial and the final stages. The inoculations were preceded by sonication as a synchronous cycle was required for this specific assay, in order to guarantee that the whole bacterial population was in exponential growth at the time-points chosen for DNA extraction (10, 20, 30 h post-infection). DNA samples were used to quantify the number of *C. trachomatis* genomes throughout real-time quantitative PCR using the ABI 7000 SDS (Applied Biosystems) as performed in a previous work (Borges et al., 2010). As for other bacteria, the doubling time (dt) was calculated using the standard formula: $dt = (t_f - t_0)/n$, where $t_f - t_0$ is the time elapsed during the period of higher growth and n is the number of bacterial generations. The n was calculated based on the formula: $n = (\log_{10}N_f/N_0)/\log_{10}2$, where N_f and N_0 are the genome copy numbers determined at the defined time-points.

To quantify the number of *C. trachomatis* plasmids, we used the same DNA samples (10, 20 and 30 h post-infection) as above, and performed absolute real-time quantification. Briefly, a 51 bp fragment of a single-copy *C. trachomatis* plasmid gene (pCTA_0008; according to the plasmid annotation of the strain A/Har13; GenBank accession number CP000052) was amplified (primers pCTA_0008_1 ATTTTTCCGGAGCGAGTTACG and pCTA_0008_2 GTACATCGGTCAACGAAGAGGTT designed using Primer Express software – Applied Biosystems) and cloned into a TOPO vector before transforming DH5α competent cells. High-copy-number plasmid DNA was purified from selected transformed-colonies, and the exact number of plasmids was measured. Cloning, transformation, and plasmid purification and quantification procedures were performed as previously described (Borges et al., 2010). Finally, the obtained plasmid copy number was divided by the corresponding number of chlamydial genomes in each DNA sample.

For the evaluation of the attachment/entry rate, we used fresh EB-enriched inocula (twin-inoculums were stored at −80 °C) to infect confluent HeLa229 cell monolayers in 6-well culture plates (multiplicity of infection of 1). After centrifuging for 1 h at 3500 rpm, plates were placed at 37 °C (5% CO_2) for 1 h. Subsequently, the inoculum was removed, the cells were washed with fresh medium (to remove EBs that were not capable to infect), and plates were again placed at 37 °C (5% CO_2) for 1 h. Cells were scraped and mechanically disrupted by sonication. DNA was extracted from both the initial inocula and the collected samples at 2 h post-infection (to ensure that no "second-generation" bacteria would be quantified) using the DNA Mini Kit (Qiagen) according to manufacturer's instructions. Finally, the number of chlamydial genomes was quantified by real-time PCR in each sample, as described above. The attachment/entry rate was then evaluated for L2/434/Bu(i), E/CS88(i), L2/434/Bu(f) and E/CS88(f) by measuring the fraction of bacteria present in the initial inoculum that effectively infected HeLa229 cells.

2.6. Mathematical modeling

Considering the mutational profile revealed in the present long-term evolution experiment, we performed mathematical modeling in order to study the selective advantages underlying variations in the frequency of mutations in evolved bacterial populations. We calculated the selective advantage (S) for a specific mutation occurring in the clone b (derived from the non-mutated clone a) as:

$$S = [(Pa_0/Pb_0) \times (Pb_n/Pa_n)]^{1/n}$$

where *Pa* and *Pb* are the percentages of the non-mutated and the mutated clone in the population (*Pa* + *Pb* = 1), respectively, in the interval of bacterial generations (0, *n*). By modeling the fixation of a single mutation, the putative existence of clonal interference (i.e., competition between clones carrying dissimilar advantageous mutations) is not taken into account, which could somehow increment the selective advantage values displayed by this model. Also, other variables such as mutation rates *per* type of mutation, which are well-studied for other microbes (Gerrish and Lenski, 1998), were not included because the association between mutant genotypes and phenotype is not straightforward in *C. trachomatis*. The present simulation also allowed us to infer if the frequency of different clones present in the initial population (which was used for full-genome sequencing) was adulterated during the short scale-up procedure from the clinical swab. Furthermore, based on the calculated mutation rates (see results), we predicted the rate of appearance of variant clones during a single *C. trachomatis* developmental cycle.

3. Results

3.1. Clonal characterization of the initial populations

We characterized the clonal diversity present in the bacterial population of the starting inocula as no *a priori* clone isolation was performed. For the prototype strain L2/434/Bu, the present evolution experiment likely constituted an expansion of a single clone as no measurable mixture of clones was detected in any genomic position. As a curiosity, we compared the obtained genome sequence with that from a same-ancestor clone fully-sequenced in 2008 (Thomson et al., 2008), and detected only two chromosomal nucleotide differences: (i) the T ↔ C in the intergenic region IGR CTL0031(*hemA*)-CTL0032(*sycE*) (chromosome position: 39,654 bp); and (ii) the non-synonymous mutation C ↔ T in the gene CTL0259 (*gatB*) (chromosome position: 326,941 bp).

Contrarily, for the clinical isolate E/CS88, the population in the initial inoculum was found to be a mixture of clones. The full-genome data exposed 23 nucleotide positions revealing clone variants in the population (Supplementary Table 2). A detailed analysis revealed a bacterial population composed by one clearly predominant clone (represented in more than 70% reads) and a non-measurable number of low-frequent variant clones. It is worth noting that from the 23 variable nucleotide positions, two of them affect the same gene (CT530/*fmt*), and three are located in genes (CT135, CT394/*hrc* and CT511/*rlpO*) where mutations had been previously found among clones from a same parental serovar D prototype strain (Sturdevant et al., 2010). In particular, the gene CT135 has been pointed out as a mutagenic hot-spot for chlamydiae (Russell et al., 2011), since multiple CT135 genotypes were found among mixed-clone populations in both *C. trachomatis* (Sturdevant et al., 2010; Suchland et al., 2008) and *Chlamydia muridarum* (Ramsey et al., 2009; Russell et al., 2011) species. Also, most of the reported polymorphisms in CT135 were single-nucleotide indel events or single inactivating mutations that prematurely truncate the predicted proteins. Remarkably, the predicted CT135 protein of the dominant clone of the strain E/CS88(i) is also truncated by a premature stop codon leading to a loss of 257 amino acids from its C terminus, which likely causes the complete loss of the protein function.

3.2. C. trachomatis growth rate

The bacterial doubling time of the strains under evaluation was estimated in order: (i) to extrapolate the number of bacterial generations enrolled during this long-term experiment; (ii) to calculate the mutation rates *per* base pair (or *per* genome) *per* replication; and (iii) to evaluate the bacterial fitness throughout the assessment of growth dynamics in the initial and final stages of the long-term *in vitro* propagation. Throughout the experiment, we estimated mean doubling times of 2.78 h (SD ± 0.56) and 2.72 h (SD ± 1.41) for the L2/434/Bu and E/CS88 strains, respectively, which are in the range interval (1.5–4 h) of previously reported doubling times for this species (Borges et al., 2010; Mathews et al., 1999; Miyairi et al., 2006; Shaw et al., 2000). Although LGV strains are generally assumed to grow faster than remainder strains, several reports have found either non-LGV strains growing as fast as the former or discrepancies in the calculated doubling time for the same LGV strain (Borges et al., 2010; Lambden et al., 2006; Miyairi et al., 2006). The large standard deviations reflect the fluctuations in the growth rates observed during the ~1-year experiment, and also bias the extrapolation of the number of generations elapsed, ranging from ~2000 to ~5000 with a mean value of 3500 generations. Although there were permanently bacterial populations under exponential growth in the continuously maintained cultures (due to the use of trypsinization as the major harvesting method), these estimates are somehow biased by the existence of non-replicating sub-populations. Nevertheless, we believed this is a rational approach considering the asynchronous *C. trachomatis* life-cycle (Shaw et al., 2000).

3.3. Evaluation of genomic alterations after long-term in vitro passaging

The whole-genome sequence data retrieved from the *C. trachomatis* cultures at end of the long-term passaging experiment were compared with those obtained from the initial inocula. Regarding the plasmid, no changes were observed in the sequences for both L2/434/Bu and E/CS88 strains. For the chromosome, neither indel or duplication events nor genomic rearrangements were observed, but we detected few single nucleotide mutations (SNPs) that were introduced in the bacterial population of both strains during laboratory propagation (Table 1). The mutations were classified according to their frequency in the population at the time of sampling: (i) fixed (present in all clones of the population); (ii) high-frequent (carried by most clones); and (iii) low-frequent (mutations at low measurable frequency).

For the prototype L2/434/Bu, we have initially propagated a single clone but the resequencing at the end of the experiment revealed a mixture of coexisting clones in the population. In fact, besides two mutations that swept to fixation (affecting the *loci* CTL0084 and CTL0103), we observed four additional chromosomal nucleotide positions variable among clones: two mutations [in the IGR tRNA-Leu/CTL0243(xerD) and CTL0486] also carried by the high-frequent clone, and two mutations (in CTL0610 and CTL0818) from clone variants at a low measurable frequency in the population. The mutations are essentially in coding regions, alter the coded amino acid, and A.T ↔ C.G transversions are overrepresented (Table 1). For the E/CS88 strain, for which we had identified one clearly predominant clone in E/CS88(i) population, we observed one single clone in the E/CS88(f) population harboring one single fixed mutation [an A.T ↔ C.G transversion affecting the IGR CT114/CT115 (*incD*)] relative to the high-frequent initial clone (Table 1). In fact, all high frequent bases of the 23 variable positions initially identified for the E/CS88(i) population (Supplementary Table 2) became fixed. This scenario is likely explained by both the extinction of the initially present low frequent clones, and the fixation of one adaptive mutation in the high-frequent initial clone during laboratory passaging. As stated above, a *Mycoplasma* contamination was detected but, due to its residual nature, it is unlikely that it constituted a competitive factor and influenced the observed evolutionary scenario. All nucleotide changes here detected resulting from the long-term laboratory

Table 1
Genomic alterations after long-term *in vitro* passaging.

Strain	Mutation location[a]	*loci*[b]	nt (aa) change[c]	Ts/Tv[d]	Frequency[e]	Putative function
L2/434/Bu						
	107093	CTL0084 (CT715)	G → C (Arg → Pro)	Tv	F	UDP-N-acetylglucosamine pyrophosphorylase Stephens et al. (1998)
	127347	CTL0103 (CT734)	A → G (silent)	Ts	F	Lipoprotein Thomson et al. (2008)
	297976	IGR tRNA-Leu/CTL0243 (*xerD*) [IGR tRNA-Leu/CT864 (*xerD*)]	C → A	Tv	H	——
	579560	CTL0486 (CT234)	A → C (Gln → His)	Tv	H	Membrane transport protein from the major facilitator superfamily Stephens et al. (1998) and Thomson et al. (2008)
	727251	CTL0610 [CT356 (*yyaL*)]	A → C (Asn → His)	Tv	L	Thioredoxin domain-containing protein Stephens et al. (1998) and Iliopoulos et al. (2003)
	946412	CTL0818 (CT555)	G → T (Ala → Ser)	Tv	L	snf2/rad54 family helicase C-terminus Iliopoulos et al. (2003)
E/CS88						
	135236	IGR CT114/CT115 (*incD*)	A → C	Tv	F	——

[a] Polymorphism location refers to the location in the reference genomes: L2/434/Bu (GenBank accession number NC_010287) and E/Bour (GenBank accession number HE601870.1).
[b] The *loci* designations are based on genome annotation of the strain L2/434/Bu (GenBank accession number NC_010287). The corresponding nomenclature in the annotated D/UW3 genome (GenBank accession number NC_000117) is also shown between parentheses. For E/CS88, only the latter was used. IGR, intergenic region with adjacent ORFs indicated.
[c] The nucleotide changes in open reading frames are presented in the 5′ to 3′ direction.
[d] Ts and Tv correspond to A.T ↔ G.C transitions and A.T ↔ C.G transversions, respectively.
[e] Three levels of the frequency of mutations in the clone-mixture population were established: (i) fixed (F): mutations carried by all clones (all reads support the mutation); (ii) high-frequent (H): mutations carried by the majority of the clones (more than 50% of reads support the mutation); and (iii) low-frequent (L): mutations at low measurable frequency (less than 50% of the reads carry this nucleotide change).

propagation had never been reported in any *C. trachomatis* strain for which full-genome sequences were already made available.

3.4. Impact of laboratory propagation on C. trachomatis fitness

While monitoring both chlamydial cultures by phase-contrast microscopy throughout the long-term experiment we observed no visible changes in the size of inclusions as well as in cell morphological features previously pointed to be indicative of differences of strains' cytotoxity, namely cell rounding, detachment, and lysis (Belland et al., 2001). We also evaluated the bacterial doubling time as it is expected to be a stable and species-specific feature under optimal growth conditions, where fluctuations in the growth rates may be indicative of fitness alterations (Andersson and Hughes, 1996). Although we observed visible fluctuations on the strains growth rate (demonstrated by variable doubling times calculated during the experiment, as described above), L2/434/Bu(f) and E/CS88(f) presented growth rates resembling those of L2/434/Bu(i) and E/CS88(i), respectively.

We also checked for variations in the number of plasmids *per* genome (Fig. 1) as the plasmid was recently pointed to play a primary role in *C. trachomatis* infectivity *in vivo* (Carlson et al., 2008). For both strains, higher plasmid copy numbers were observed during the exponential phase of the life-cycle, and no significant differences were detected in the number of plasmids *per* genome between the initial and final cultures ($P = 0.268$ and $P = 0.075$ for L2/434/Bu and E/CS88, respectively; two-way ANOVA test), indicating that the plasmid load was likely not affected by the long-term *in vitro* maintenance of *C. trachomatis* strains.

Finally, as measure of the attachment/entry rate, we have also compared the percentage of chlamydial organisms that effectively infected HeLa229 cells using bacterial inocula from both the initial and final cultures (Fig. 2). For the historical strain L2/434/Bu, we detected similar levels of attachment/entry rate for L2/434/Bu(i) and L2/434/Bu(f) ($P = 0.868$; *t*-Student test), whereas E/CS88(f) displayed an increase of attachment/entry rate of about 20% when compared to E/CS88(i) ($P = 0.057$; *t*-Student test).

3.5. Calculation of mutation rate and mathematical modeling

The *C. trachomatis* mutation rate *per* base pair *per* replication (μ_{bp}) were 3.84×10^{-10} for E/CS88 and 1.10×10^{-9} for L2/434/Bu, which correspond to a mutation rate *per* genome *per* replication (μ_g) of 0.0004 and 0.0011, respectively (i.e., 0.04–0.11% of the new clones generated after one single replication in a bacterial population will carry a new mutation). By extrapolating these mutational dynamics to a single developmental cycle (Fig. 3), it could be virtually expected that up to 0.25% of the generated clones will be variant. It is worth noting that this value is underestimated as there is no way to take into account mutations that do not reach a detectable frequency (including deleterious mutations, which rapidly disappear) (Herring et al., 2006).

We also performed mathematical modeling to numerically plot the selective advantage of one mutated clone against the number of generations needed to reach a given frequency in the population (Fig. 4A). In fact, for an asexual evolving bacterial population under strict neutrality, it is intuitively expected that one clone carrying a new mutation will not rise in frequency unless the mutation confers advantage over its ancestor and other lineages. For a specific final frequency, the selective advantage is a decreasing function of the number of generations. Also, for a fixed number of generations, higher final frequencies implicate higher selective advantages of the mutated clone. We further applied this model to assess if the clone frequencies in the E/CS88(i) population (revealing a dominant clone with >70% frequency) reflect those contained in the clinical swab. In fact, we cannot discard that deviations in these frequencies could have been introduced during the minimal culture scale-up (four *in vitro* passages) performed to obtain sufficient DNA for full-sequencing of the initial inoculum. The computational simulations presented in the Fig. 4B revealed that if the dominant clone was a minor frequent clone in the population of the original swab, by assuming for instance 5%, it would need an overwhelming selective advantage of more than 1.14 to reach (after solely four *in vitro* passages) the empirically observed 70% frequency at the time of sequencing (Fig. 4B). This value seems somehow unrealistic considering the model highlighted in

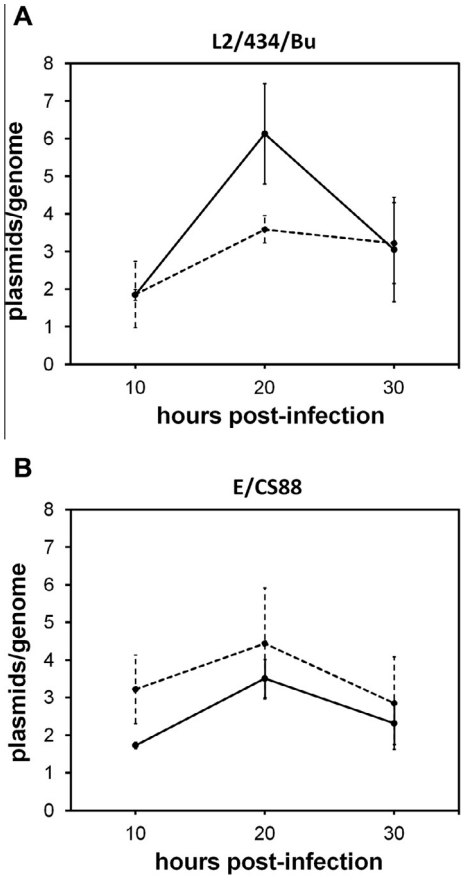

Fig. 1. Impact of long-term laboratory passaging on the *C. trachomatis* plasmid load. The number of plasmids *per* genome was evaluated before and after the laboratory propagation of the strains L2/434/Bu (A) and E/CS88 (B). The plasmid load was quantified throughout the developmental cycle (10, 20 and 30 h post-infection) of the strains by absolute real-time quantitative PCR. Solid and dashed lines represent the strains in the initial and final stages of the evolution experiment, respectively. The results (mean ± SD) are based on two independent experiments.

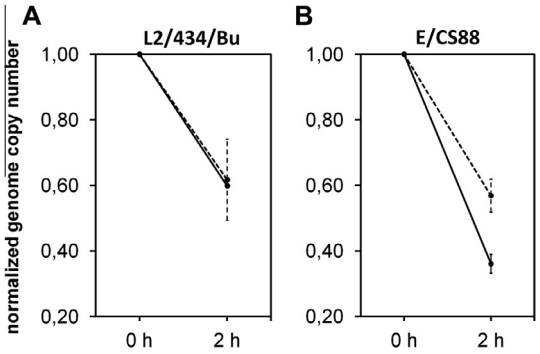

Fig. 2. Evaluation of the attachment/entry rate of strains before and after their long-term *in vitro* propagation. The attachment/entry rate was evaluated by calculating the fraction of bacteria present in the initial inoculum (measured at 0 h) that effectively infected HeLa229 cells (measured at 2 h post-infection). The assay was performed before (solid line) and after (dashed line) the long-term laboratory propagation of the strains L2/434/Bu (panel A) and E/CS88 (panel B). The number of *C. trachomatis* genomes in each sample was quantified through absolute real-time quantitative PCR. The values (mean ± SD) at 2 h post-infection were normalized to the number of genomes present in the initial inoculum (0 h), which were arbitrarily set to 1. The ~20% increase of attachment/entry rate observed for E/CS88(f) when compared to E/CS88(i) was not statistically significant ($P = 0.057$; *t*-Student test). The results are based on two independent experiments.

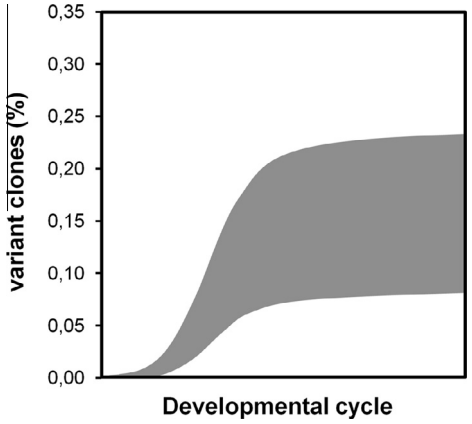

Fig. 3. Prediction of the rate of appearance of variant clones. The graph shows the percentage of the variant clones that likely arise during one single *C. trachomatis* developmental cycle (about seven bacterial generations). Values were extrapolated from the empirically estimated mutation rate *per* genome *per* replication (between 0.0004 and 0.0011) and are independent of the initial population size.

Fig. 4A. Also, this would reflect an opposite scenario from the one observed in the present long-term experiment. In fact, although up to 0.25% of variant clones are expected after each *C. trachomatis* developmental cycle (Fig. 3), only a single advantageous clone was observed for the E/CS88(f) population after thousands of generations, which sustain the well-assumed scenario where a random mutation have a considerable lower probability of being advantageous than neutral or deleterious in evolving bacterial populations (Sniegowski et al., 2000). In an alternative hypothesis, a dominant nature-rescued clone (hypothetically presented in the bacterial population in the clinical swab) would have undergone a complete extinction throughout the minimal scale-up process due to deleterious effects of the *in vitro* environment. However, this is also hardly explained considering the likely optimal physiological conditions underlying cell culture. Accordingly, we believe that the clone frequencies in the E/CS88(i) population reflect those contained in the clinical swab. Nevertheless, the only way to really prove this would be the immediate use of the clinical sample for sequencing (which is unfeasible due to insufficient number of bacteria in the swab).

4. Discussion

It is well known that pathogens may adapt to laboratory culture conditions, which implies that sequences obtained from laboratory-maintained strains may contain artifacts that introduce bias when analyzing evolutionary landscapes. This has been observed, for instance, in human influenza virus, where "laboratory-derived" mutations (mainly in the receptor binding pocket of the haemagglutinin protein) were found to account for up to 8% of all non-silent changes (Bush et al., 2000), as well as in the gp120 envelope glycoprotein of HIV-1 (Sullivan et al., 1998), and in the VP1 capsid protein of foot-and-mouth disease virus (Martínez et al., 1997). In *C. trachomatis*, the fixation of mutations in the *in vitro* environment was experimentally demonstrated through the induction of selective pressure by antibiotic exposure (Binet and Maurelli, 2005; Kutlin et al., 2005; Rupp et al., 2008; Sandoz et al., 2012; Suchland et al., 2005). Previous studies that attempted to evaluate if *in vitro* propagated *C. trachomatis* strains are genetically unstable focused on analyzing the stability of the traditional typing gene – *ompA* (over 20 laboratory passages) (Stothard et al., 1998) and of loci from multi locus typing systems (over 72 laboratory passages) (Labiran et al., 2012), and found no variation on sequence data retrieved before and after the isolates being expanded in tissue

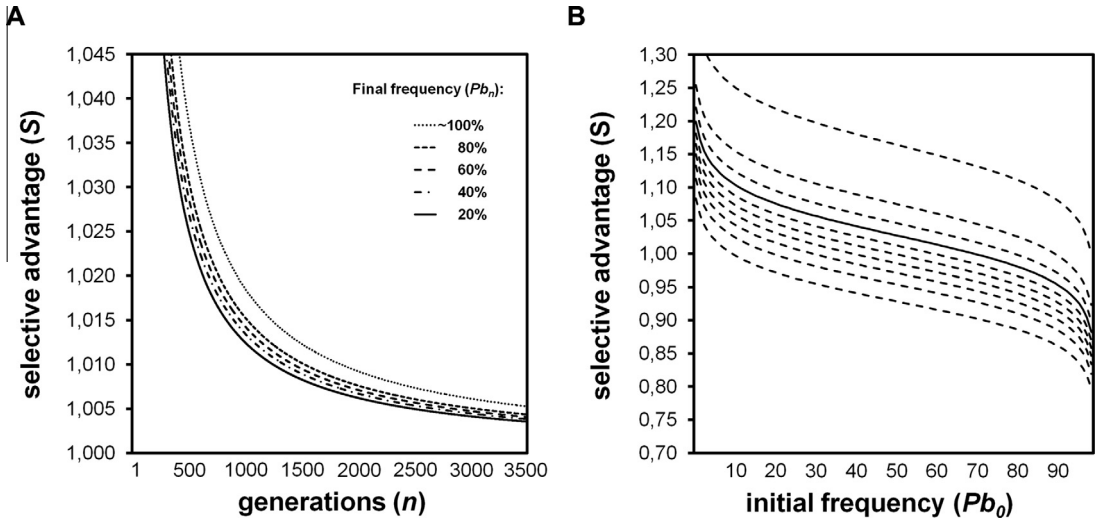

Fig. 4. Mathematical modeling of fluctuations in clone frequency. (A) The number of generations (n) needed by a mutated clone to reach a given final frequency (Pb_n) is governed by the selective advantage (S) conferred by a beneficial mutation. Higher selective advantages implicate lower number of generations needed, or higher frequencies reached (for a fixed number of generations elapsed). This simulation assumes a population size of 10^6 at the time the advantageous mutation occurred. (B) This simulation reflects the selective advantages underlying putative fluctuations in the initial clones' frequency (Pb_0) during the minimal culture scale-up procedure (four *in vitro* passages) (n = 30). The solid line corresponds to the empirically observed frequency (70%) of the abundant clone in the E/CS88(i) population, whereas the dashed lines represent final frequencies (Pb_{30}) from 10% (lower plot) to ~100% (upper plot).

culture. Those studies specifically aimed to evaluate the stability of genotyping markers, so the limited number of genomic regions analyzed (up to nine) and the short-term duration of the experiments prevent the knowledge of the real extent of genomic alterations that may arise due to laboratory adaptation.

In the present study, we analyzed the impact of long-term *in vitro* propagation on the genome of two *C. trachomatis* strains by fully-sequencing their genomes before and after about 250 *in vitro* passages in tissue culture (up to 3500 bacterial generations). We observed four SNPs (six if one considers additional two carried by low frequent variant clones) for the historical prototype strain L2/434/Bu and one SNP for the clinical isolate E/CS88 (Table 1), which corresponds to an estimated mutation rate in the range of 3.84×10^{-10}–1.10×10^{-9} mutations *per* base pair *per* generation, a value that fits previously estimates for other bacteria (Andersson and Hughes, 1996; Drake et al., 1998). Due to the obligate intracellular life-style of *C. trachomatis*, which is characterized by small replicating populations frequently subjected to transmission bottlenecks, we would expect to observe some genetic drift. In fact, we had previously detected traces of Muller's ratchet mutations (Felsenstein, 1974; Muller, 1964) as a result of *C. trachomatis* evolutionary speciation (Borges et al., 2012). Despite the present evolution experiment enrolled an unprecedented evaluation of thousands of chlamydial generations, this "evolutionary scale" still may be too modest to allow the observation of such evolutionary phenomena *in vitro*. For instance, a similar study in *Escherichia coli* detected a drift signature solely after 20,000 generations (Barrick et al., 2009). Considering that this free-living bacterium replicates about eight-times faster than *C. trachomatis*, such evaluation would be unworkable for this bacterium whose growth is host-cell dependent.

We believe that the present experiment encompasses a number of bacterial replications that may represent decades of cell culture work enrolling a specific strain of a common chlamydial research laboratory. Indeed, cultures are commonly performed from stocks of multiple twin-aliquots of enriched inocula, which are used over several years without being further re-inoculated for generating additional aliquots (and thus, without introducing substantial bacterial generations in the initial population). Therefore, our results pointing to a considerable genomic stability *in vitro* suggest that the *in vivo*-derived genetic diversification is not significantly compromised by the "modest" number of laboratory passages performed throughout decades in most chlamydial research laboratories. Accordingly, when we compared the full-genome sequence of our L2/434/Bu(i) with that of L2/434/Bu from another research laboratory, which was fully-sequenced five years ago (Thomson et al., 2008), we only found two nucleotide differences, although these clones enclose necessarily distinct histories of laboratory maintenance.

We performed computer simulations to study mutational fitness, which suggest that alterations in frequencies of the clone variants in the population are possible only under non-neutral selective pressures. Indeed, in a random mutational scenario exclusively mathematically ruled (i.e., in the absence of evolutionary forces driving selective sweeps), any frequent mutation in the beginning of the experiment will keep its frequency at the end. Thus, we speculated that the mutations observed in the present study are advantageous, which is clearly supported by both the mathematical modeling (Fig. 4) and also the huge amount of literature data concerning the evaluation of laboratory adaptation of microbes, as comprehensively compiled in a recent review (Dettman et al., 2012). Although phenotype variations are hard to trace due to the lack of straightforward methods for genetically manipulating *C. trachomatis* chromosome, a careful inspection of the observed mutations (Table 1) points in that direction. For L2/434/Bu: (i) all but one mutation are A.T ↔ C.G transversions, which we estimated to be about 2.6 fold more unlikely than A.T ↔ G.C transitions in the *C. trachomatis* diversification [based on previously published data (Borges et al., 2012)]; (ii) all but one mutation are located in coding regions and four of them cause amino acid replacement; and (iii) one of the fixed mutations (non-synonymous) affects CTL0084 that codes for a protein (UDP-N-acetylglucosamine pyrophosphorylase) with transferase activity, which was found to be among the gene functional categories more prone to undergone advantageous mutations in laboratory evolution studies in *E. coli* (Conrad et al., 2011); (iv) another non-synonymous mutation involves a thioredoxin coding gene (CTL0610); thioredoxins are part of the gene expression regulation apparatus

(Meyer et al., 2009), which is known to be a preferred selection target of adaptive mutations (Cooper et al., 2003; Dettman et al., 2012). For E/CS88, the final population had no diversity indicating that the mutation likely conferred a selective advantage, and swept to fixation during the evolution experiment (Fig. 4). Moreover, the mutation is a A.T ↔ C.G transversion, and it is located in the intergenic region upstream of the gene encoding the inclusion membrane protein D (CT115/*incD*), which is an effector protein (Subtil et al., 2005) recently suggested to play a role in subverting the non-vesicular lipid transport machinery of the host cell (Derré et al., 2011). However, besides its putative location in the regulatory region of a virulence gene, there is no additional experimental evidence suggesting that the mutation is advantageous. Finally, for both strains, the mutations that became prevalent had to "survive" the drift imposed by the consecutive bottlenecks created during serial passaging, and also the putative occurrence of clonal interference (Gerrish and Lenski, 1998). Nevertheless, we cannot discard that some of these mutations can be just neutral mutations that rose in frequency either by their occurrence in the same clone carrying a beneficial mutation or by recombination (genetic hitchhiking) (Elena and Lenski, 2003). We cannot be conclusive about this because the evaluation of the frequency of selective sweeps would demand sequencing the strains at intermediate stages of the long-term experiment.

In contrast to L2/434/Bu, the initial inoculum of E/CS88 was constituted by a mixture of clones, where the dominant clone (which additionally acquired the described mutation) became 100% frequent after laboratory propagation, and the remainder evolved to extinction. Remarkably, only the latter carried the virulence gene CT135 in its active form. Thus, we concluded that CT135 is superfluous for *C. trachomatis* survival *in vitro*. This strongly corroborates the findings of Sturdevant et al. (2010) that implicated CT135 in the pathogenesis of *C. trachomatis* urogenital infections while suggesting its dispensability *in vitro*.

Globally, we point to a slow adaptation of *C. trachomatis* to the *in vitro* environment, which, in the present study, was governed by minor genomic alterations. Despite the low number of observed mutations, we cannot discard that they may be sufficient for altering fitness. In fact, marked changes in phenotype were shown to be mediated by very few mutations in studies using *E. coli* (Herring et al., 2006; Lee and Palsson, 2010), *Saccharomyces cerevisiae* (Zeyl, 2005), and the influenza virus (Taubenberger et al., 2005). Commonly, the phenotype-genotype relationships of mutations are studied through genetic knockout assays, which are still giving the first steps in *Chlamydia* (Kari et al., 2011; Mishra et al., 2012; Nguyen and Valdivia, 2012; Song et al., 2013; Wang et al., 2011). Consequently, we performed humble approaches for evaluating the impact of laboratory propagation on *C. trachomatis* fitness, and the results were not helpful. Indeed, despite fluctuations in the growth rates during the experiment, we did not observe considerable differences between initial and long-term propagated strains. Also, we found no significant variation in the number of plasmids *per* bacterium after the intensive serial passaging of both strains in tissue culture (Fig. 1), which is intriguing in the light of previous studies suggesting that the plasmid is not required for *C. trachomatis* growth *in vitro* (Carlson et al., 2008; Farencena et al., 1997; Peterson et al., 1990). However, additional studies in *Chlamydiaceae* suggested that the plasmid is important for the infectivity *in vitro* (O'Connell and Nicks, 2006; Russell et al., 2011), indicating that the basis for plasmid maintenance by this species still remains to be clarified. We speculate that an even longer evolutionary experiment would be needed to evaluate the putative *in vitro* extinction of the *C. trachomatis* plasmid, although a recent study in *Borrelia burgdorferi* sensu lato (Biškup et al., 2011) showed the plasmid loss after just less than 10 *in vitro* passages. Regarding the assay for evaluating the attachment/entry rate (Fig. 2), although we observed an increase in the fraction of bacteria that effectively infected the HeLa229 cells after the E/CS88 strain has been long-term propagated, the result was not statistically significant ($P = 0.057$; *t*-Student test). We have no reasonable explanation for this difference, besides the fact that the E/CS88(i) population constituted a mixture of competing clones (with expected variable fitness), and that the E/CS88(f) harbored a putative adaptive mutation.

Although the present assessment is genome-based, it is worth noting that gene expression changes may also account for adaptation of evolving bacteria (Conrad et al., 2011). Besides the discreet genomic changes that were observed in the present study, we cannot rule out the existence of significant transcriptomic alterations on the course of *C. trachomatis* laboratory propagation. In fact, previous laboratory evolution experiments with other bacteria showed that different endpoints of evolution have distinct underlying gene expression states (Cooper et al., 2003; Fong et al., 2005; Stoebel et al., 2009; Vannucci et al., 2012).

Finally, the demonstration of a remarkable degree of parallel evolution between *Pseudomonas fluorescens* and its phage Phi2 (Paterson et al., 2010) makes us wonder if the scenario observed in our study would be mirrored in the absence of such strict interplay between the host cell and the parasite. In fact, for *Coxiella burnetii* [also an obligate intracellular bacterium for which it was already developed a culture medium that allows growth in the absence of host cells – axenic growth (Omsland et al., 2009)], an interesting work found that virulent strains displayed attenuated growth, less ability to induce splenomegaly, and less elicitation of antibody responses when infecting mice after long-term axenic passaging (Kersh et al., 2011). On behalf of the recent advances in the development of an axenic system for *C. trachomatis* culture (Omsland et al., 2012), it would be interesting to evaluate if the host-cell free *in vitro* propagation of *C. trachomatis* strains somehow shapes their genomic or transcriptomic signatures.

As concluding remarks, although phenotype inferences are hard to trace, our genome-based approach highlighting very few nucleotide changes suggests that *in vitro* propagation of *C. trachomatis* strains acts as an "evolutionary freezer" of the *in vivo* genomic diversity rather than an effective modifier of the evolutionary landscape.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2013.03.035.

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