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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.