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Research paper

VNTR diversity in *Yersinia pestis* isolates from an animal challenge study reveals the potential for *in vitro* mutations during laboratory cultivation

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ABSTRACT

Underlying mutation rates and other evolutionary forces shape the population structure of bacteria in nature. Although easily overlooked, similar forces are at work in the laboratory and may influence observed mutations. Here, we investigated tissue samples and *Yersinia pestis* isolates from a rodent laboratory challenge with strain CO92 using whole genome sequencing and multi-locus variable-number tandem repeat (VNTR) analysis (MLVA). We identified six VNTR mutations that were found to have occurred *in vitro* during laboratory cultivation rather than *in vivo* during the rodent challenge. In contrast, no single nucleotide polymorphism (SNP) mutations were observed, either *in vivo* or *in vitro*. These results were consistent with previously published mutation rates and the calculated number of *Y. pestis* generations that occurred during the *in vitro versus* the *in vivo* portions of the experiment. When genotyping disease outbreaks, the potential for *in vitro* mutations should be considered, particularly when highly variable genetic markers such as VNTRs are used.

1. Introduction

Molecular variation can provide insight into the structure and history of bacterial populations. It may be generated by mutation, horizontal gene transfer, and/or recombination. Natural selection and genetic drift may then act on that molecular variation, affecting the frequency and distribution of different alleles in bacterial populations. The relative importance of these evolutionary forces will vary depending on the bacterial species and will affect the types and patterns of molecular variation within a species. Likewise, even within a single species, each type of molecular variation may have varying mutation rates and be differentially affected by recombination, selection and drift, and should be interpreted accordingly (Keim et al., 2004). Thus, experiments aimed at measuring mutation rates, rates of recombination or the impact of selection and drift can provide significant insights into the proper interpretation of observed molecular variation in bacterial populations.

Yersinia pestis, the etiologic agent of plague, is a highly successful recently emerged pathogen with established enzootic foci in Asia, Africa, and the Americas (Keim and Wagner, 2009). Overall, this pathogen exhibits relatively low genetic diversity due to its recent emergence and ecology (Keim and Wagner, 2009; Morelli et al., 2010). However, both single nucleotide polymorphism (SNP) analysis and multi-locus variable-number tandem repeat (VNTR) analysis (MLVA) have successfully been used to genetically discriminate among strains. Indeed, various studies of *Y. pestis* phylogeography using SNPs and/or VNTRs have revealed a consistent pattern of the spread of just one or a few genotypes followed by subsequent differentiation at local (Girard et al., 2004; Vogler et al., 2013), regional (Girard et al., 2004; Vogler et al., 2011), and worldwide scales (Cui et al., 2013; Morelli et al., 2010). This pattern is the result of both the underlying mutation rates generating variation and other evolutionary forces related to plague ecology.

Plague ecology can be characterized by alternating enzootic and epizootic cycles, both involving rodents and fleas affected by the disease (Gage and Kosoy, 2005). The enzootic phase is relatively cryptic and still poorly understood, but serves to maintain *Y. pestis* in the environment long-term through low level cycling in reservoir (*i.e.*, enzootic) hosts that are thought to be either resistant to plague or have a high replacement rate. In contrast, the epizootic phase is characterized by massive population die-offs of highly susceptible epizootic hosts that serve to amplify and spread *Y. pestis* (Biggins and Kosoy, 2001; Cully and Williams, 2001; Perry and Fetherston, 1997). These enzootic and epizootic cycles differ in their rates of bacterial replication per unit time, leading to a highly variable molecular clock rate across the *Y. pestis*

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phylogeny with more SNPs and branching events in lineages associated with more epidemic disease. Similarly, high rates of fixation may be observed when *Y. pestis* successfully becomes established in new geographic areas due to founder effects (Cui et al., 2013).

In contrast to the real-time molecular clock rate, which appears to vary considerably (Cui et al., 2013; Wagner et al., 2014), the underlying cellular mutation rates in *Y. pestis* are assumed to be relatively constant. First, there is little evidence of mutator strains in *Y. pestis* (Cui et al., 2013). Second, a comparison between *in vitro* VNTR mutation rates measured in laboratory serial passage experiments and *in vivo* mutation rates predicted from the mutations observed during a rodent epizootic showed remarkable congruence (Girard et al., 2004). That said, there have been no direct attempts to examine *Y. pestis* mutation rates *in vivo*. In this study, we examine *in vivo* mutation rates, their relationship to *in vitro* mutation rates, and the repercussions of laboratory culturing steps on observed mutations by characterizing *Y. pestis* mutations observed during an experimental challenge of wild-caught Gunnison's prairie dogs (*Cynomys gunnisoni*) with strain CO92.

2. Materials and methods

2.1. Challenge experiment

Sixty wild-caught Gunnison's prairie dogs were challenged with a fully virulent *Y. pestis* strain (CO92). Full experimental details of the challenge and sample collection have been described previously (Busch et al., 2013). Briefly, prairie dogs were randomly chosen for challenge with 50, 5,000 or 50,000 CFUs of *Y. pestis* and monitored daily for illness during a 30 day experiment. Moribund prairie dogs were humanely euthanized when they were identified (Day 8–17) and any surviving animals were euthanized at the end of the experiment. Liver, lung and spleen tissue samples were flash frozen and later used to isolate *Y. pestis* from those prairie dogs who died of plague or would have died of plague if not euthanized ($n = 24$); *Y. pestis* was not obtained from any surviving prairie dogs. All challenge experiment related work was approved by the Arizona Game and Fish Department (AZGFD), the Institutional Animal Care and Use Committee (IACUC) at Northern Arizona University (NAU) (field collection of prairie dogs, tissue sample *Y. pestis* isolations, and molecular analyses), and the IACUC at US Geological Survey National Wildlife Health Center (USGS-NWHC) (challenge experiment and tissue sample collection) (Busch et al., 2013).

2.2. Strain isolation and screening

Y. pestis was cultured from each tissue sample by cutting off a piece of partially thawed tissue and streaking the internal tissue surface onto TSA with 5% sheep blood. A single colony from this streak was then used to streak a bacterial lawn on a second TSA with 5% sheep blood agar plate. All plates were incubated for 48 h at 28 °C. The *Y. pestis* lawn was roughly divided into fourths with ~3/4 used to create glycerol stocks to be preserved at −80 °C and another ~1/4 used to extract DNA using a Qiagen DNeasy Blood and Tissue Kit (Valencia, CA). In all, 70 DNA extracts were screened with two real-time PCR assays to confirm the presence of *Y. pestis* (Hinnebusch and Schwan, 1993; Radnedge et al., 2001; Stevenson et al., 2003) and then analyzed with a 43-locus MLVA (Girard et al., 2004). These DNA extracts included the CO92 strain used to infect the prairie dogs and 2–3 isolates derived from each *Y. pestis*-positive prairie dog (liver, lung and spleen isolates). Two *Y. pestis*-positive prairie dogs lacked lung isolates due to cannibalization of their lungs by other prairie dogs before removal from the common housing room (Busch et al., 2013) and one *Y. pestis*-positive prairie dog's spleen isolate was negative for *Y. pestis* and so was not further analyzed (Fig. 1, Table S1).

2.3. Amplicon sequencing

PCR product for VNTR marker M61 was sequenced from one of the *Y. pestis* isolates from prairie dog #9 that exhibited an atypical M61 allele size (Fig. 1, Table S1). Amplicon sequencing was performed using forward and reverse M61 primers (Klevytska et al., 2001), the BigDye

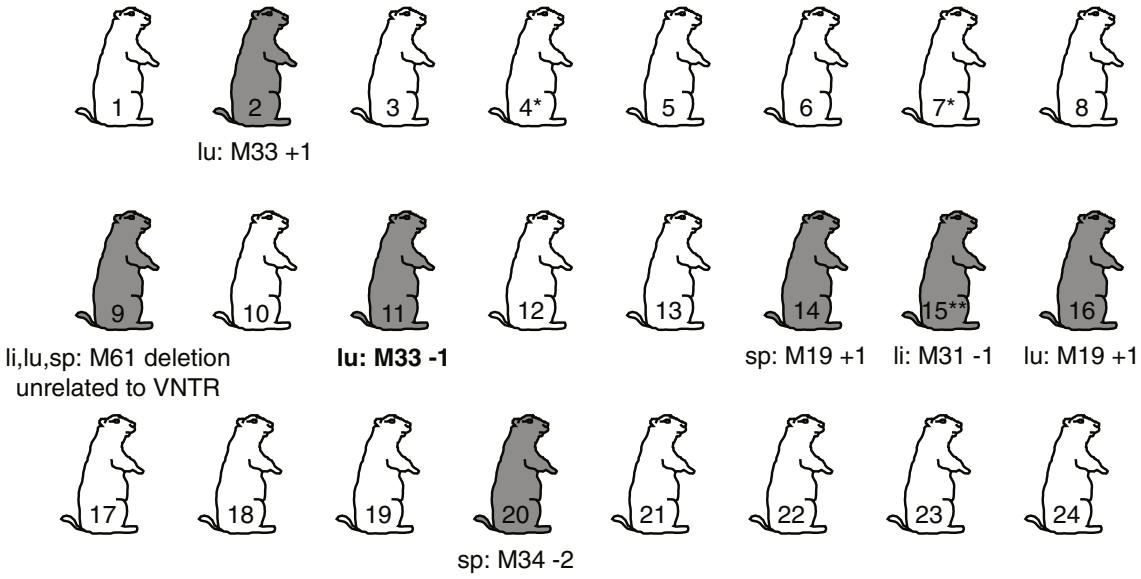

Fig. 1. Schematic of VNTR mutations among *Yersinia pestis* isolates from 24 prairie dogs that succumbed to experimental infection with plague. Three *Y. pestis* isolates, isolated from liver (li), lung (lu), and spleen (sp) tissues, respectively, were recovered from most of the prairie dogs. For three of the prairie dogs, only two *Y. pestis* isolates were obtained. The lack of a third isolate was due to either cannibalization of the lungs (*) or failure to obtain an isolate from the spleen (**). Prairie dogs from which mutant *Y. pestis* isolates were obtained are colored gray. For each of these prairie dogs, the tissue sample(s) with mutant isolates, *Y. pestis* MLVA-43 marker with the mutation and type of mutation (generally, + or − for an insertion or deletion, respectively, followed by the number of repeats involved in the mutation) are indicated. The bold text indicates the single *Y. pestis* mutant that did not exhibit both the parental and mutant alleles (*i.e.*, a double allele) at the mutated marker.