Destabilization of tetrancleotide repeats in
\emph{Haemophilus influenzae} mutants lacking RnaseH1
or the Klenow domain of Poll

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ABSTRACT

A feature of \emph{Haemophilus influenzae} genomes is the presence of several loci containing tracts of six or more identical tetrancleotide repeat units. These repeat tracts are unstable and mediate high frequency, reversible alterations in the expression of surface antigens. This process, termed phase variation (PV), enables \emph{H.influenzae} to rapidly adapt to fluctuations in the host environment. Perturbation of lagging strand DNA synthesis is known to destabilize simple sequence repeats in yeast and \emph{Escherichia coli}. By using a chromosomally located reporter construct, we demonstrated that the mutation of an \emph{H.influenzae} rnhA (encoding RnaseH1) homologue increases the mutation rates of tetrancleotide repeat repeats ~3-fold. Additionally, deletion of the Klenow domain of DNA polymerase I (PolI) resulted in a ~35-fold increase in tetrancleotide repeat-mediated PV rates. Deletion of the PolI 5’>3’ exonuclease domain appears to be lethal. The phenotypes of these mutants suggest that delayed or mutagenic Okazaki fragment processing destabilizes \emph{H.influenzae} tetrancleotide repeat tracts.

INTRODUCTION

Replication of double-stranded DNA genomes is semi-discontinuous with DNA replication being continuous on the leading strand and discontinuous on the lagging strand. Lagging strand DNA synthesis initiates from RNA primers of 8–12 nt in length, proceeds for ~100–2000 nt and terminates in response to signals that include the 5’ end of an RNA primer (prokaryotes) or DNA flaps (eukaryotes) (1,2). Processing of these DNA fragments, termed Okazaki fragments, requires removal of the RNA primer/DNA flap, filling of the gap between the two DNA fragments by DNA synthesis and DNA ligation. In eukaryotes, perturbation of Okazaki fragment processing can destabilize simple sequence repeats (3–5).

Okazaki fragment processing in \emph{Escherichia coli} is initiated by RnaseH1, which removes or shortens the RNA primer (6). Polymerase I (PolI) then extends the 3’ end of the upstream Okazaki fragment, displaces a single nucleotide of the 5’ end of the downstream fragment, cleaves the displaced single-strand DNA and thus creates a substrate for DNA ligase (7). PolI has three structural domains with different activities: 5’>3’ exonuclease, 3’>5’ exonuclease and 5’>3’ DNA polymerase (8). The latter two domains form the Klenow fragment. In \emph{E.coli}, polA (i.e. polI) mutants lacking either polymerizing (polA1) or 5’>3’ exonuclease (ΔpolA, F’ Klenow) activity exhibit elevated mutation frequencies for dinucleotide repeat tracts (9). While activation of an SOS response was partially responsible for the increase, these results indicate that accurate Okazaki fragment processing is required to maintain the stability of dinucleotide repeat tracts in \emph{E.coli}.

\emph{Haemophilus influenzae} is a common commensal of the upper respiratory tract of humans. Characteristically, the genomes of this bacterial species contain multiple long tetrancleotide repeat tracts (10,11). Long repeat tracts with alternate unit sizes (e.g. mono or dinucleotide repeats) are infrequent. These tetrancleotide repeat tracts have high-mutation rates and are responsible for PV, i.e. a high frequency of reversible switches in expression, of surface antigens such as lipopolysaccharide epitopes and haemoglobin–haptoglobin binding proteins (12,13). The mutation rates of these repeat tracts are influenced by the number of repeats in the tract but are not subjected to control by mismatch repair, recA-mediated recombination or transcription-coupled repair (14,15). Interestingly, an insertion/deletion mutation in the \emph{H.influenzae} polI gene destabilized tetrancleotide, but not dinucleotide, repeat tracts independently of activation of an SOS response suggesting a critical role for Okazaki fragment processing in determining the stability of repeats in this bacterial species (14). In this study, we have extended these

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observations by constructing a mutation in an rnhA (encoding RnaseHI) homologue and a deletion of the PolI Klenow domain of H. influenzae, and show that both these mutations destabilize tetraneucleotide repeat tracts.

MATERIALS AND METHODS

Strains

H. influenzae strain RM118, termed Rd herein, is a derivative of strain KW-20 (16). Strains RdGAZ38R and RdGΔZ17R were described previously (15). H. influenzae strains were grown in brain heart infusion (BHI) supplemented with either haemin (10 μg/ml) and NAD (2 μg/ml) for liquid media or Levinthal supplement for solid media. E. coli strains DH5α or GM48 were used to propagate plasmids and were grown in Luria–Bertani broth supplemented with ampicillin (100 μg/ml), kanamycin (50 μg/ml), tetracycline (12 μg/ml) or chloramphenicol (30 μg/ml).

Construction of mutations in poll and rnhA of H. influenzae

Here, we describe the construction of a number of plasmids that were used in attempts to inactivate genes or to delete specific regions of genes on the H. influenzae chromosome (see Figure 1 for a depiction of the inserts in these plasmids and Table 1 for the DNA sequences of the oligonucleotides utilized for the cloning of these inserts). The designations of these constructs are as follows: a gene preceded by Δ indicates that the gene was inactivated by the insertion of an antibiotic cassette (deletion of a portion of a gene may also occur in these constructs); a gene followed by a Δ and then a domain(s) name indicates the deletion of a particular domain(s) of a gene and that the antibiotic cassette is in an adjacent gene (in these constructs it is assumed that the other domains of the protein will be expressed). The designations for the domains of poll (see above and Figure 1) are as follows: 5′ to 3′ exonuclease, 5′>3′ exo; 3′ to 5′ exonuclease, 3′>5′ exo; 3′ to 5′ exonuclease and DNA polymerase, Klen, Klen.

In construct pUCΔpoll (14), 666 bp of the H. influenzae poll gene were deleted and replaced by a tetracycline cassette. Plasmid pUCΔpoll-large was constructed in order to extend this deletion into the poll 5′>3′ exo domain. This plasmid was made by amplifying from H. influenzae strain Rd chromosomal DNA a fragment that included sequences upstream of poll and part of the 5′>3′ exo domain, using primers POL9E and POL8H, which, respectively, have EcoRI and HindIII sites at the 5′ end. This fragment was digested with EcoRI and HindIII and used to replace the EcoRI–HindIII fragment of pUCΔpoll (Figure 1a). Recombination of the insert of this plasmid into the H. influenzae chromosome would result in deletion of the entire 3′>5′ exo domain and part of the 5′>3′ exo domain.

Plasmid, pUCpollΔKlen, was constructed to enable deletion of the entire Klenow domain and efficient expression of the 5′>3′ exo domain. The entire 5′>3′ exo domain and some upstream sequences were amplified with primers POL10RV (binds downstream of the Poll 5′>3′ exo domain) and POL9E. The former primer has an EcoRV site at the 5′ end and inserts two 5′TAA stop codons in frame with the poll reading frame, enabling efficient termination of translation. This fragment was digested with EcoRV and EcoRI and used to replace the EcoRV/EcoRI fragment of plasmid pUCΔhi0855 (14). Recombination of the insert of this plasmid into the H. influenzae chromosome would result in deletion of the entire Klenow domain and the 5′ end of the downstream gene, hi0855.

Figure 1. Construction of mutations in poll and rnhA of H. influenzae. (a) Representations of the native H. influenzae poll locus (top diagram) and of deletions/insertions in this locus that were constructed in plasmids are shown. The ‘hi’ numbers are taken from the annotation of the Rd genome sequence in the TIGR Microbial database (www.tigr.org). Genes are represented by open boxes and non-coding DNA by a thick dark line. The three domains of Poll are shown as filled boxes: dots, 5′>3′ exonuclease domain; filled box, 3′>5′ proof-reading exonuclease domain; and vertical stripes, polymerase domain (this analysis is based on data available in the TIGR Microbial database). The direction of translation is indicated by arrows. Plasmids pUCΔpoll and pUCΔhi0855 were described previously (14). Antibiotic cassettes were inserted into these plasmids in either the HindIII (pUCΔpoll and pUCΔpoll-large) or EcoRV sites. The parentheses indicate the poll domains that could be expressed in H. influenzae constructs derived using plasmids pUCpollΔKlen, pUCpollΔS>3′exo and pUCpollΔS>5′exo/3′>5′ exo. (b) The construction of an insertional mutation in rnhA is shown. The top diagram represents the native locus and indicates the positions of two primers used to clone a 1700 bp fragment containing the rnhA gene. A tetracycline cassette (tet^R) was inserted into a Bcl site located in the centre of rnhA.
A full-length clone of the *H. influenzae* polI gene was constructed by amplifying chromosomal DNA with primers POL9E and POL4B (binds within polI downstream of the HindIII site), digesting the product with EcoRI and HindIII (a native site within polI), and using this fragment to replace the EcoRI/HindIII fragment of pUCΔpolI. The resultant plasmid was digested with HindIII and BamHI and the larger fragment was ligated to two fragments generated from plasmid was digested with HindIII and BamHI and the larger fragment was ligated to two fragments generated from pUC aptos. The resultant plasmid, pUCpolI-full, contains the entire polI gene and promoter region plus an interrupted version of gene *hi0855*.

Plasmids, pUCpolII53>3exo and pUCpolII53>3exol 5>3exo, were constructed to enable deletion of either the 5>3exo domain alone or both the 5>3 and 3>5exo domains. DNA fragments lacking these domains were generated by PCR amplification using plasmid DNA of pUCpolI-full and either POL12KLEN (binds at the beginning of the PolI 3>5exo domain) and POL7B (binds downstream of hi0855) or POL13POL (binds at the beginning of the PolI polymerase domain) and POL7B primers. These fragments were digested with NcoI (present at the 5’ ends of POL12KLEN and POL13POL) and EcoRV. PCR amplification of chromosomal DNA was then performed with primers POL14E (binds upstream of polI and has an EcoRI site at the 5’ end) and POL11NC (binds adjacent to but upstream of the native polI start codon and has an NcoI site at the 5’ end). This fragment was digested with EcoRI and NcoI and together with one of the NcoI–EcoRV fragments was used to replace the EcoRI/EcoRV fragment of pUCpolI-full. Recombination of the inserts of the resultant plasmids, pUCpolII53>3exo and pUCpolII53>3exol 3>5exo>3>5exo, into the *H. influenzae* chromosome would result in the expression of either the Klenow or polymerase domains of PolI, respectively.

To enable selection for these mutations in *H. influenzae*, antibiotic cassettes were then inserted into each of these plasmids. A tetracycline cassette [derived from pHTV1 (17)] was inserted in the HindIII site of pUCΔpolI-large and into the EcoRV site of pUCpolIΔKlen, pUCpolII53>3exo and pUCpolII53>3exo>3>5exo (note that in the latter three constructs hi0855 will be inactivated). In addition, a chloramphenicol cassette [derived from pACYC184 as described in (18)] was inserted into the EcoRV site of pUCpolII53>3exo and pUCpolII53>3exo>3>5exo.

Plasmid, pUCahrA-tet, was constructed to permit inactivation of the *H. influenzae* rnhA gene. The rnhA gene and flanking regions were amplified by PCR from chromosomal DNA of *H. influenzae* strain Rd using primers RNASE1B and OMP2EXT. This product was digested with BamHI and EcoRI and ligated to pUC19 cut with the same enzymes. This plasmid was transformed into *E. coli* strain GM48 to allow restriction with BclI, which is sensitive to methylation. A tetracycline cassette was ligated into the unique BclI site, in the centre of rnhA, of this plasmid.

These plasmids were linearized with either SalI or BamHI and transformed into competent *H. influenzae*. Transformants were selected on BHI plates containing 4 µg/ml tetracycline or 2 µg/ml chloramphenicol. Transformants were checked by PCR amplification using primers spanning the deletion/antibiotic cassette insertion sites and by Southern-blot analysis using probes, specific for polI or rnhA, which were generated by PCR amplification and labelled using the dioxigenin system (Boehringer Mannheim).

### Growth and cell division assays

Growth rates and numbers of colony forming units (c.f.u.) were measured for strains grown in liquid culture. Cultures (5 ml) were inoculated with 0.3 ml of an overnight culture of each strain and grown to an optical density (OD) at 490 nm of 0.4. Cultures were then standardized by diluting back to an OD of 0.1. Optical densities were measured at 490 nm every 2 h. Serial dilutions of samples, taken at different intervals throughout the growth curve, were plated and used to estimate the total number of c.f.u. Some samples were also examined by phase contrast microscopy. Doubling times were estimated by plotting log N (where N is the number of c.f.u.; OD490 units) against time and fitting a trendline to the linear portion of the growth curve.

### Phase variation rate assays

PV rates and mutational patterns were determined as described previously (15). Briefly, *H. influenzae* mutants were transformed with linearized DNA of plasmids pGAZ38R, pGAZ17R and pGAZAT20, and transformants were isolated on BHI plates containing 10 µg/ml of kanamycin. These plasmids recombine into the *H. influenzae* chromosome and express a phase variable Mod–LacZ fusion protein whose PV is driven by, respectively, 38 5’AGTC, 17 5’AGTC or 20 5’AT repeats. PV rates were determined for two transformants of each combination of mutant strain-reporter construct. Serial dilutions of multiple colonies of a strain were plated on BHI plates containing 40 µg/ml of Xgal

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**Table 1. Oligonucleotides utilized in construction of plasmids**

| Primer name | Sequence (5’ to 3’) | Description of binding site | Position of binding site |
|-------------|---------------------|----------------------------|--------------------------|
| POL8H       | CAAAGCTTGTACCCCACTAGTGC | Internal to polI            | 421 to 441               |
| POL9E       | CCGAATTCCTTTAGCCAACTGACCC | Upstream of polI           | +181 to +201              |
| POL10Rv     | CCAATATCTTTAATTTTACCGGCACCTTGATC | Internal to polI           | 947 to 968               |
| POL11NC     | GGCATATGAACTCTGTTTTAAGC | Upstream of polI           | 3 to +20                  |
| POL12KLEN   | CCCCCATGCGCCCTAAATTTCAACGGATCG | Internal to polI           | 1588 to 1608              |
| POL13POL    | CCCCCATGCGAAACTGGAATTTGC | Upstream of polI           | +844 to +864             |
| POL14E      | CGGAGCTTCAGCTGAATCACCTCC | Upstream of rnhA         | –937 to –918              |
| RNASE1B     | CGGAATTCCTTTAGCTTGTTTACTC | Downstream of rnhA         | +222 to +245              |

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(5-bromo-4-chloro-3-indoyl-β-d-galactopyranoside) and these plates were used to estimate the total number of variants and cells in a colony. PV frequencies were calculated from these values and then PV rates were derived using the median frequency either according to Drake (19) or to Saunders et al. (20). Alterations in repeat tracts were determined by PCR amplification from parental and variant progeny colonies using fluorescently labelled primers which span the repeat tract. The sizes of PCR products were determined by electrophoresis using an ABIprism 377 autosampler and analysis of gels with the ABI GeneScan 3.1 program (PerkinElmer).

The increase in ON-to-OFF switching of strain Rd polI-Klen was separated into increases in shifts of differing unit sizes using the observed proportions of each shift size (i.e. 1, 2 or >2 units). The increase in deletions and insertions for each unit size was then calculated using the observed ratio of deletions:insertions in ON-to-OFF switching (i.e. a ratio of 30:1 implies that 1/31 of the 1 unit shifts are insertions). These values were then used to estimate the increases in OFF-to-ON switching (i.e. the increase in −1 switching is the combined values for the increase in −1, +2, −3 and +1 shifts).

Statistics

Statistical analyses were performed with the program Instat 2.0. For PV frequencies, comparisons of the PV frequencies for a pair of strains were performed using a Mann–Whitney non-parametric rank sum test. For proportions of insertions and deletions, pairs of strains were compared using a 2 × 2 contingency table and a Fisher’s exact test where the two-sided P-value tests the null hypothesis that the proportion of deletions and insertions is identical in each strain.

RESULTS

Construction of a deletion of the polI Klenow domain and inactivation of rnhA of H. influenzae

Mutant Rd polI was constructed by interrupting the H. influenzae polI gene with an antibiotic cassette inserted into the region encoding the Klenow domain of PolI (see pUCpolI in Figure 1). While this mutant exhibited an elevation of tetrancleotide repeat-mediated PV rates (14), it was unclear whether this increase was due to complete loss of PolI activity (i.e. both 5’>3’ exonuclease and polymerase activity) or just the absence of PolI polymerase activity. The deletion in pUCpolI was therefore extended to include part of the 5’>3’ exo domain. The resultant plasmid, pUCpolI-large, was used to transform H. influenzae strain Rd. All of the transformants obtained had undergone a single cross-over event and retained an intact polI gene. This result suggested that inactivation of the entire polI gene was lethal as observed for E. coliΔpolA mutants grown on rich media (21).

We then constructed a series of plasmids that would permit deletion of the entire Klenow domain (pUC polIIKlen), the 5’>3’ exo domain (pUC polII5’>3’exo) or the 5’>3’ and 3’>5’ exo (pUC polII5’>3’exo3’>5’exo) domains (see Figure 1). A tetracycline cassette was inserted into an artificial EcoRV site in hi0855, the gene downstream of polI whose inactivation was known not to destabilize tetrancleotide repeat tracts (14). Transformants were obtained with each construct but only those transformed with pUC polIIKlen had undergone double cross-over events and retained the deletion. These transformants were designated Rd polIIKlen. This result demonstrated that deletion of the Klenow domain of H. influenzae PolI was possible but suggested that deletion of the 5’>3’ exo domain was lethal.

A major function of PolI is processing of Okazaki fragments. To test whether inactivation of other proteins involved in this process also destabilizes tetrancleotide repeat tracts, a mutation was constructed in the H. influenzae rnhA homolog. The rnhA gene and flanking region was cloned from H. influenzae strain Rd and a tetracycline cassette was inserted into a native BclI site in the centre of the gene. This construct was used to transform H. influenzae strain Rd and transformants were generated containing an interrupted rnhA gene. These transformants were designated RdΔrnhA.

Growth characteristics of mutants

Growth of the PolI-Klenow and RnaseHI mutants was examined for Rd polIIKlen, RdΔrnhA and Rd in liquid media (Figure 2a). The mutants exhibited doubling times of 51 (±0.8) and 76 (±3.3) min, respectively, whereas the doubling time of strain Rd was 50 (±4.6). These data indicate that the loss of RnaseHI activity has a much greater effect on growth than loss of Klenow activity. Filament formation was investigated by plating samples from different points along the growth curves and calculating the numbers of c.f.u./ml present in the culture. To enable comparison between the different time points these values were divided by the number of OD units (Figure 2b). Strain Rd exhibited a small increase in the number of c.f.u. per OD unit as the cultures progressed into stationary phase, possibly indicating that during early stages of the growth curve DNA replication/cell growth occurred faster than cell division. In three cases, these curves also exhibited a significant reduction in the number of c.f.u. after overnight growth likely due to cells dying. Both the Rd polIIKlen and RdΔrnhA mutants exhibited large reductions in the number of c.f.u. per OD unit with the average figures being 8- or 14-fold lower, respectively, than the average figure for strain Rd (1.6 × 109 c.f.u./OD490 unit, ±8 × 108). Examination of the mutants by phase contrast microscopy revealed the presence of filaments of varying sizes in the liquid cultures (data not shown), which may partly or wholly explain the observed decreases in c.f.u. per OD unit. Similarly, reductions in the number of c.f.u. per colony were also noted when the mutants were grown on solid media, once again indicating the presence of filaments.

Phase variation rates of polII mutants

PV rates were investigated by inserting a lacZ reporter construct containing 5’AGTC tetrancleotide repeats into the chromosome of the mutant strains. Median PV frequencies were determined for each mutant using reporter constructs containing 38 or 17 repeats (Table 2). Rates were determined for both directions of switching i.e. ON-to-OFF (blue to white colonies) and OFF-to-ON (white to blue colonies). Note that in these constructs there is only one ON reading frame for every two OFF reading frames and that the majority of mutations in strain Rd involve loss or gain of a single repeat unit. Thus, an ON construct with 38 repeats will usually switch to OFF variants containing either 39 or 37 repeats. Meanwhile...
OFF-to-ON switching takes two forms. In $-1$ switching of strain Rd, the majority of ON variants are generated by loss of a repeat unit (e.g. 39 to 38 repeats) while in $+1$ switching gain of a repeat unit is the commonest event (e.g. 37 to 38 repeats). Obviously ON variants can also be generated by larger events but these occur infrequently (e.g. 37 to 35 or 32 repeats).

Mutation rates were derived for these constructs either according to Drake (19), to facilitate comparisons to our previous data, or to Saunders et al. (20) (Table 2). This latter method includes a calculation of the back-mutation rate and may, therefore, provide a more accurate estimation of PV rate. Table 2 indicates that the Drake equation can underestimate high-mutation rates by up to 1.3-fold and can overestimate low-mutation rates by up to 1.5-fold but in general provides a reasonably accurate estimation of PV rate.

PV rates for Rd$\text{polI}$$\Delta$Klen were increased relative to strain Rd by 31- to 40-fold for ON-to-OFF switching and 30- to 36-fold for $-1$ OFF-to-ON switching but only 5- to 17-fold for $+1$ OFF-to-ON switching. These data indicated that tetranucleotide repeat tracts are destabilized in Rd$\text{polI}$$\Delta$Klen and the greater effect on OFF-to-ON switching of $/C_0$ versus $+1$ constructs suggested that there was a higher proportional increase in deletions relative to insertions.

Analyses of the types of mutations occurring in variant colonies of Rd$\text{polI}$$\Delta$Klen reporter constructs were performed and compared to analyses performed on strain Rd and Rd$\Delta$hi0855 (Figure 3). For ON-to-OFF switching in Rd$\text{polI}$$\Delta$Klen, no insertions were seen in a construct with 17 5$'$AGTC repeats while the ratio of deletions to insertions for a 38 repeat construct was 30:1. This figure was significantly higher than
the ratios of 2.4:1 and 2.3:1 observed with reporter strains of Rd and RdΔnh0855, respectively, containing similar numbers of repeats (by Fisher’s exact test P-values of 0.016 and 0.006 were obtained). For −1 OFF-to-ON switching in which a −1 deletion produces an ON phenotype, the ratio was slightly higher for RdpolΔKlen, 21:1 (combined data for 36 and 15 5’AGTC repeats), than for strain Rd, 7:1 (combined data for 24 and 18 5’AGTC repeats), but the difference between these ratios was not significant (P = 0.56). For +1 OFF-to-ON switching in which a +1 insertion produces an ON phenotype, the ratio for RdpolΔKlen was 5:1 (for both 37 and 16 5’AGTC repeats), which was significantly higher than the ratio for

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**Table 2. Influence of pol-klen and rnhA mutations on H.influenzae PV rates**

| Relevant genotype | Direction of switching | Repeat no./type | Mutation frequency (× 10⁻³) | Mutation rate (× 10⁻⁴) | Repeat no./type | Mutation frequency (× 10⁻³) | Mutation rate (× 10⁻⁴) | Repeat no./type | Mutation frequency (× 10⁻³) | Mutation rate (× 10⁻⁴) | Repeat no./type | Mutation frequency (× 10⁻³) | Mutation rate (× 10⁻⁴) |
|-------------------|------------------------|----------------|-----------------------------|------------------------|----------------|-----------------------------|------------------------|----------------|-----------------------------|------------------------|----------------|-----------------------------|------------------------|
| wt                | ON-to-OFF              | 38/AGTC        | 4.07e                       | 4.3 (7.25–3.04) [1.0]  | 37/AGTC        | 2.65e                       | 2.64 (2.9–1.6) [1.0]  | 36/AGTC        | 37/AGTC        | 3.04 (2.45–1.57) [1.0]  | 3.04 (2.45–1.57) [1.0]  | 30/AGTC        | 36.6 (35.0–37.2) [1.0]  |
| Δpol-klen         | ON-to-OFF              | 35/AGTC        | 173.8                       | 131.24 (145.0–109.5) [30.5] | 166.74 | 37/AGTC        | 36.6                       | 45.85 (39.6–26.1) [17.4] | 39.31 |
| ΔrnhA             | ON-to-OFF              | 38/AGTC        | 12.74                       | 13.85 (21.2–10.54) [2.5] | 11.31 | 37/AGTC        | 6.21                       | 6.18 (7.16–5.72) [2.3]  | 4.96 |
| wt                | OFF-to-ON (+1)         | 17/AGTC        | 1.02e                       | 1.14 (1.48–0.6) [1.0]  | 0.79 | 18/AGTC        | 0.67e                       | 0.75 (1.02–0.37) [1.0]  | 0.5  |
| Δpol-klen         | OFF-to-ON (+1)         | 17/AGTC        | 50.4                        | 45.1 (382.4–39.7) [39.6] | 44.36 | 16/AGTC        | 4.37                       | 3.75 (5.11–2.5) [5.0]  | 3.11 |
| ΔrnhA             | OFF-to-ON (+1)         | 17/AGTC        | 4.72                        | 5.14 (6.39–3.42) [4.5]  | 3.92 | 18/AGTC        | 2.85                       | 3.16 (5.91–1.46) [4.2]  | 2.32 |
| wt                | OFF-to-ON (-1)         | 20/AT           | 1.79e                       | 1.89 (2.25–1.08) [1.0]  | 1.38 | 22/AT           | 1.27e                       | 1.39 (1.78–1.06) [1.0]  | 0.98 |
| ΔrnhA             | OFF-to-ON (-1)         | 20/AT           | 2.28                        | 2.52 (4.74–1.63) [1.3]  | 1.83 | 19/AT           | 1.26                       | 1.64 (2.03–0.78) [1.2]  | 1.07 |

**Figure 3. Influence of pol-klen and rnhA mutations on the types of alterations occurring in 5’AGTC tetrancleotide repeat tracts.** Repeat tracts from phase variants were amplified by PCR and either sequenced or sized by a gene scan protocol. Alterations were classified as insertions or deletions of 1, 2 or >2 repeat units and the number of each mutational type was then represented as a percentage of the total number of tracts analysed. The genotype of each strain is indicated below the columns (wt is strain Rd) and the direction of switching above the columns. The number of repeats in the parental strain is indicated in parentheses while the total number of tracts analysed is in curly brackets. Data for wt 17, wt 37 and wt 24/18 were published previously (15).

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Mutation rates in these columns were derived from the median frequency by the method of Drake (19). In the majority of cases median frequencies were determined from the analysis of 14 or more colonies. For the following strains only eight colonies were analysed: Δpol-klen 17 5’AGTC; Δpol-klen 37 5’AGTC; ΔrnhA 18 5’AGTC; ΔrnhA 19 5’AGTC; ΔrnhA 18 5’AT. Finally the data for Δpol-klen 36, 16 and 15 5’AGTC were derived from 6, 3 and 5 colonies, respectively. Numbers in curly brackets are 95% confidence intervals calculated according to Kokoska et al. (3). Numbers in parentheses and italics are the range of observed values. Numbers in square brackets are the fold increase relative to a parental strain with a reporter construct of equivalent repeat type and number.

Mutation rates in these columns were derived according to Saunders et al. (20) using the median frequencies.

Values as reported previously by De Bolle et al. or Bayliss et al. (15,30).
strains Rd, 1:4 (for 37 S'AGTC, P = 0.017), and RdΔhi0855, 1:33 (for 37 S'AGTC, P = 0.0001).
The relative proportions of 1:2>2 repeat units shifts (i.e. 1 repeat unit shifts are the combined values for −1 and +1 shifts, etc.) was similar between RdpollΔKlen and strain Rd, 79.5:15.5:5 and 78.5:17:4.5, respectively (derived from the combined data for 35/17 and 38/17 repeat reporter constructs), indicating that the sizes of alterations in repeat number had not changed. Using these values and a 30:1 ratio of deletions to insertions for ON-to-OFF switching in RdpollΔKlen, it can be calculated (see Materials and Methods) that the ~35-fold increase in ON-to-OFF switching was due to increases of 33.8-fold for deletions and 1.2-fold for insertions. This analysis would predict that −1 OFF-to-ON switching would increase ~30-fold while +1 OFF-to-ON switching would increase ~8-fold. These values are similar to the observed values, supporting the notion that the elevated PV rates of RdpollΔKlen were due to a major increase in the number of deletions.

**Phase variation rates of rnhA mutants**

PV rates of reporter constructs containing either tetraneucleotide (5'S'AGTC) or dinucleotide (5'AT) repeats were examined in rnhA mutants of H.influenzae (Table 2). PV rates mediated by tetraneucleotide repeats were elevated for both directions of switching by ~2.4-fold for long tracts (37 and 38 repeats) and ~4.4-fold for short tracts (17 and 18 repeats). These differences were significant as shown by the non-overlapping 95% confidence intervals and P-values of <0.001 obtained in Mann-Whitney (non-parametric) rank sum tests comparing PV frequencies of parental and mutant strains. The increase (1.9-fold) for an RdΔrnhA mutant containing 16 S'AGTC repeat tracts was not significant (P-value of 0.17) but this may due to the comparison being made with an 18 S'AGTC reporter construct, an equivalent strain Rd reporter construct not being available. Contrastingly, PV rates for the dinucleotide repeats were elevated only 1.3- and 1.4-fold for ON-to-OFF and OFF-to-ON switching, respectively. The majority of these increases were not significant as shown by overlapping 95% confidence intervals and P values of 0.1 and 0.61 for the 20 and 19 S'AT repeat constructs, respectively. The construct with S'AT 18 repeats had a significantly (P-value of 0.009) elevated PV rate but the importance of this result is unclear as this rate was higher than that of an RdΔrnhA mutant reporter construct containing 19 S'AT repeats.

The types of alterations occurring in repeat tracts of RdΔrnhA mutants were also examined. The pattern of mutations occurring in both the S'AGTC (Figure 2) and S'AT (data not shown) repeat tracts were similar to that observed with strain Rd.

**Lethality of H.influenzae S'>3' exonuclease mutation**

Transformation with the pUCpollΔ5'>3'exo-tet and pUCpollΔ5'>3'exo/3'>S'exo-tet plasmids (see above) frequently generated transformants that had undergone recombination between the tetracycline cassette and the deletion (i.e. between the Ncol and EcoRV sites, see pUCpollΔ5'>3'exo and pUCpollΔ5'>3'exo/3'>S'exo in Figure 1). In order to force recombination to occur at the ends of the constructs (i.e. upstream of the Ncol site and downstream of the EcoRV site), we inserted a chloramphenicol cassette into the EcoRV site and used these constructs to transform the tetracycline-resistant RdpollΔKlen mutants described above. This transformant lacks the DNA sequences between the EcoRV site and the deletion (compare pUCpollΔKlen and pUCpollΔ5'>3'exo, see Figure 1), such that recombination can occur only at the ends of the plasmids. Only a small number of transformants were obtained and all these transformants had undergone only single cross-over events. The ability of RdpollΔKlen to incorporate foreign DNA into the chromosome was tested by transformation with a plasmid carrying an unrelated H.influenzae gene (i.e. lgtC). The mutant exhibited a transformation frequency similar to that of strain Rd (data not shown). These results provide a further indication that deletion of the H.influenzae PolI S'>3' exonuclease domain is lethal.

**DISCUSSION**

Multiple cis- and trans-acting factors govern the stability of microsatellites in both prokaryotes and eukaryotes (22). Microsatellites or simple sequence repeats mediate high frequencies of reversible changes in the expression of virulence factors in a number of bacterial species (13,23–25). To fully understand the contribution of these repeats to bacterial adaptation, an in-depth analysis of the factors controlling the occurrence of mutations in these repeats is required. In this study, we have demonstrated that deletion of the entire PolI Klen domain destabilizes tetraneucleotide repeats ~35-fold while inactivation of RnaseH, another enzyme involved in processing of Okazaki fragments, also destabilizes tetraneucleotide repeats although to a lesser extent. In addition, we provide evidence that the S'>3' exonuclease domain of PolI is required for viability.

**Poll polymerase activity is not required for H.influenzae viability**

In E.coli, viable polA mutants lacking either the S'>3' exonuclease or Klenow domains have been generated (21). Survival of these mutants demonstrates that the exonuclease and polymerase activities of Poll are not essential for replication of the E.coli genome and that other enzymes/proteins can complement these activities. The failure to construct a S'>3' exonuclease deletion mutant of H.influenzae may suggest that this activity of PolI is essential for viability of this bacterial species. It should be noted, however, that the E.coli exonuclease mutant was constructed by first expressing the Klenow domain on an F plasmid and then deleting the entire polA gene. Interestingly, it was possible to construct a viable H.influenzae mutant lacking the entire Klenow domain of Poll. H.influenzae differs from E.coli in that it lacks homologs of polB, dinB and umuC/umuD (26,27) whose products are specialized DNA polymerases (i.e. PolIII, PolIV and PolV) that could be responsible for survival of the E.coli polAI mutant, which is known to be deficient in the Klenow domain. Thus, unless H.influenzae encodes an additional DNA polymerase not detectable by homology to currently known families of DNA polymerases, polymerization of the 1.8 Mb genome of this bacterial species is performed by PolIII alone. Notably, this includes the DNA polymerization required for Okazaki fragment joining.
Growth characteristics of \textit{polI-klen} and \textit{rnhA} mutants

While the growth rates of \textit{H.influenzae polIΔKlen} mutants were similar to the parental strain, the mutant cells were forming filaments (Figure 2). This suggests that growth and cell division were uncoupled which in part may result from perturbation of DNA replication. Filament formation may also be a result of induction of an SOS response. \textit{H.influenzae} has a LexA-inducible operon similar to that of \textit{E.coli} although lacking a sulA homolog (W.A. Sweetman, E.R. Moxon and C.D. Bayliss, manuscript in preparation), which is responsible for SOS-induced filament formation in \textit{E.coli} (28). \textit{H.influenzae rnhA} mutants also exhibited filamentous growth but with a slightly more severe phenotype (~14 cells/filament) and a reduction in growth rate (Figure 2). This result is surprising and suggests either that an inability to shorten the RNA primers at the ends of Okazaki fragments engenders a severe block to replication of the genome or that RnaseHI is required for other functions important for growth [e.g. initiation of DNA replication (6)].

\textbf{PV rates of \textit{polI-klen} and \textit{rnhA} mutants}

Previously, we constructed a mutation in the \textit{H.influenzae polI} gene and demonstrated that this mutation elevated tetranucleotide, but not dinucleotide, repeat-mediated PV rates by ~40-fold (14). This mutation involved partial deletion of the Klenow domain and insertion of an antibiotic cassette into the \textit{H.influenzae polI} gene. Thus, it was unclear whether expression of the 5′ portion of the PolII protein, encompassing the 5′>3′ exonuclease domain, was perturbed and whether any residual expression of the PolI polymerase domain was retained. The \textit{polI-klen} mutant described in this study resolves these issues by inserting artificial stop codons at the end of the exonuclease domain of \textit{polI}, allowing efficient translation of this fragment, and deleting the entire Klenow domain so no residual PolI polymerase activity can be produced.

Tetranucleotide PV rates were elevated ~35-fold in Rd\textit{polIΔKlen} indicating that loss of PolII DNA polymerization activity alone is responsible for destabilization of these repeats. The data presented herein indicates that this instability is due to a major increase in the numbers of deletions. It has been proposed that deletions could result from the following pathway: spontaneous or UvrD-mediated displacement of the 5′ end of the downstream Okazaki fragments located within the repeat tract, removal of the RNA primer, re-annealing of the DNA flap adjacent to the 3′ end of the upstream Okazaki fragment and ligation of the DNA ends without DNA polymerization (see Figure 4). Note that this process produces a deletion whereas insertions would result from loops forming in the displaced DNA flap and would require DNA synthesis prior to ligation (14). One unclear step was the identity of the proteins responsible for the removal of ribonucleotides at the 5′ end of the downstream Okazaki fragment. We now propose that these nucleotides are removed by cleavage of the DNA flaps by the 5′>3′ exonuclease activity contained in the N-terminal PolII fragment that is retained in the Rd\textit{polIΔKlen} mutant (Figure 4). A similar elevation of deletions and frameshift mutations has been noted for \textit{E.coli polA} mutants deficient in the Klenow domain (29). Nagata \textit{et al.} (29) proposed that mismatch bulges in the template strand are recognized by the Klenow domain of PolII and processed by the 3′>5′ exonuclease activity of this domain. However, the mechanistic basis for recognition of these mismatches was not elucidated.

The destabilization of tetranucleotide repeats by mutations in \textit{polI} implicated Okazaki fragment processing as having a major influence on the stability of such repeats. In order to further investigate the role of this process, a mutation was constructed in \textit{rnhA}, which encodes the enzyme responsible for shortening of RNA primers during completion of lagging DNA synthesis. Rd\textit{ΔrnhA} mutants exhibited a significant increase in instability of tetra- but not dinucleotide repeats as observed for Rd\textit{ΔpolI}. However, the increase was only ~3-fold and there was no increase in the number of deletions.
relative to insertions, indicating that the mutational process was mechanistically different in RdΔmha mutants. The increased PV rate of these mutants may be due to persistence of Okazaki fragments resulting in more time for the formation of DNA flaps which would then re-anneal resulting in a higher frequency of displacements of repeats in both the template and nascent DNA strands and thus a higher rate of PV (Figure 4). Alternatively, flap formation may be required for the removal of RNA primers leading to a higher frequency of displacement of repeats but with no bias towards deletions as PolI can efficiently polymerize the gaps between fragments. The absence of an effect on dinucleotide repeat tracts may be due to correction of additional mutations in these tracts by MMR.

In summary, the Klenow fragment of PolI is a major factor controlling the stability of tetrancleotide repeats in \textit{H. influenzae} while RnaseH is a minor factor. Thus, accurate Okazaki fragment processing may be an important factor for preventing slippage in a subset of prokaryotic simple sequence repeats.

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