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RecJ, ExoI and RecG are required for genome maintenance but not for generation of genetic diversity by repeat-mediated phase variation in *Haemophilus influenzae*

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Abstract

High levels of genetic diversity are generated in *Haemophilus influenzae* populations through DNA repeat-mediated phase variation and recombination with DNA fragments acquired by uptake from the external milieu. Conversely, multiple pathways for maintenance of the genome sequence are encoded in *H. influenzae* genomes. In *Escherichia coli*, mutations in single-stranded-DNA exonucleases destabilise tandem DNA repeats whilst inactivation of *recG* can stabilise repeat tracts. These enzymes also have varying effects on recombination. Deletion mutations were constructed in *H. influenzae* genes encoding homologs of ExoI, RecJ and RecG whilst ExoVII was refractory to mutation. Inactivation of RecJ and RecG, but not ExoI, increased sensitivity to irradiation with ultraviolet light. An increase in spontaneous mutation rate was not observed in single mutants but only when both RecJ and ExoI were mutated. None of the single- or double-mutations increased or decreased the rates of slippage in tetranucleotide repeat tracts. Furthermore, the exonuclease mutants did not exhibit significant defects in horizontal gene transfer. We conclude that RecJ, ExoI and RecG are required for maintenance of the *H. influenzae* genome but none of these enzymes influence the generation of genetic diversity through mutations in the tetranucleotide repeat tracts of this species.

Keywords: Phase variation; Tandem DNA repeats; Microsatellites; Repeat instability; Genome maintenance; Exonuclease; *H. influenzae*

1. Introduction

Haemophilus influenzae (*Hi*) is a Gram-negative, obligate commensal of the upper human respiratory tract with the potential to cause diseases such as meningitis, septicaemia, epiglottitis, pneumonia and otitis media. Long microsatellites (i.e. tandem DNA repeat tracts) are present in the promoter or 5′-coding regions of several *Hi* contingency loci whose products contribute to the virulence and disease associated with this bacterial pathogen [1]. Mutations in these microsatellites enable *Hi* populations to switch between genetic variants with alternate phenotypes, a process referred to as phase-variation (PV) and usually affecting surface proteins or proteins that modify surface structures. This localised hypermutation occurs within the context of multiple pathways that prevent mutations occurring within the majority of the *Hi* genome [2,3].

Multiple pathways and genes are involved in genome maintenance. Mismatch repair (MMR) is one major determinant of genome stability. The *Hi* MMR system comprises four genes; *mutS*, *mutL*, *mutH* and *dam* [4]. These genes exhibit sequence and functional homology with the MMR genes of *Escherichia coli* (*Ec*). Indeed the *Hi* MutS, MutL and MutH proteins can replace the functions of their cognate *Ec* proteins [5]. The exonucleases required for *Hi* MMR have not however been characterised. Four single-stranded-DNA (ss-DNA) exonucleases are redundantly involved in *Ec* MMR such that only deletion of all four enzymes produces an increase in mutation rate [6]. DNA cleavage is initiated from a 3′-end by ExoI and ExoX,

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from a 5′-end by RecJ, and from both 5′ and 3′ ends by ExoVII [7–10]. These exonucleases also have functions in other DNA repair and recombination pathways. RecJ can assist RecBCD-promoted recombination but also promotes recombination from DNA breaks in combination with a DNA helicase and survival of UV damage [11–13]. ExoVII is similarly required for survival of UV damage [14]. Contrastingly, inactivation of ExoI results in only minor defects in survival of UV damage and DNA recombination. However, inactivation of this enzyme in combination with mutations in other exonucleases can significantly increase UV sensitivity, which phenotype can be complemented by over-expression of ExoX [10,14]. Thus, *Ec* exhibits significant redundancy in the exonuclease components required for repair of certain types of DNA damage.

The determinants of the mutability of microsatellites have been widely studied in two organisms, *Hi* and *Neisseria meningitides* (*Nm*), in which repetitive DNA is the major mechanism of PV. All but one of the *Hi* phase variable genes contain repeat units of four or more nucleotides whilst there is a preponderance of mononucleotide repeats in the phase variable genes of *Nm* [1]. Various factors are known to determine the mutation rates of the tetranucleotide repeats present within the *Hi* genome [15–17]. Notably perturbation of DNA replication, through mutations in *polI* and *rnaseHI*, destabilise these *Hi* repeat tracts suggesting that inhibition of DNA replication-associated processes may affect the stability of repeat tracts [18]. Critically, inactivation of the *Hi* MMR system does not destabilise tetranucleotide repeat tracts but does increase the mutation rates of dinucleotide repeat tracts present in reporter constructs [4,17]. The MMR genes, *mutS*, *mutL* and *uvrD*, are the major determinants of the stability of *Nm* mononucleotide repeats [19–21]. Recently, Morelle et al. [22] demonstrated that inactivation or over-expression of ExoVII altered the mutation rates of mononucleotide repeat tracts in *Nm*. The role of exonucleases in the stability of longer repeat units has not been studied in either of these organisms.

The *Ec* genome contains few microsatellites of significant length. This organism has, however, been widely utilised as a model organism in which to study the mutability of repetitive DNA, including the trinucleotide repeats responsible for human neurodegenerative diseases. As with *Hi* and *Nm*, *Ec* MMR is required for the stability of repeats of one to three nucleotides but not of four or more nucleotides [23,24]. Feschenko et al. [25] reported that three of the *Ec* MMR-associated exonucleases, namely, ExoI, ExoX and RecJ, play a role in stabilizing tandem repeats of 100 bp, independent of their role in MMR. Recently, inactivation of *Ec recG* was shown to alter the stability of trinucleotide repeat tracts [26]. *Ec* RecG is a structure-specific DNA helicase, which is involved in re-initiation of DNA synthesis of stalled replication forks [27,28]. *Ec recJ* also facilitates re-initiation of DNA replication [29]. These results suggested, therefore, that the MMR-associated exonucleases, possibly acting through restart of stalled replication forks, might be determinants of the stability of repeat tracts in *Hi*.

The *Hi* strain Rd genome sequence contains homologs of *recG* and of genes encoding three of the *Ec* exonucleases, but not *exoX*. Mutations were constructed in each of these genes in order to investigate their roles in genome maintenance and phase variable gene expression in *Hi*. No single mutation exerted a significant effect on PV rates but mutations in *recJ* and *recG* impaired survival of DNA damage whilst inactivation of ExoVII appears to be lethal.

2. Experimental procedures

2.1. Bacterial strains and growth conditions

Hi strain RM118, a serotype d isolate termed strain Rd herein, is a derivative of strain KW-20, which was used for the genome-sequencing project [30]. Strains RdGΔZ38R, RdGΔZ17R and RdGΔZAT20 contain a translational fusion between *mod*, a phase variable methyltransferase, and *lacZ* in which expression of *lacZ* is controlled by a repeat tract of 38 5′-AGTC, 17 5′-AGTC or 20 5′-AT repeats, respectively. These strains have been reported previously [16] and were used to create reporter strains for each of the mutations examined in this study (Table 1). *Hi* strains were grown at 37 °C in brain heart infusion (BHI) supplemented either with haemin (10 μg/ml) and NAD (2 μg/ml) in liquid media or Levinthal's reagent (10%) on solid media. Antibiotic supplements of kanamycin (10 μg/ml) or tetracycline (4 μg/ml) were added to select mutant strains as appropriate.

Ec strain DH5α was used for propagation of plasmids and was grown at 37 °C in Luria-Bertani broth supplemented with kanamycin (50 μg/ml) or tetracycline (12 μg/ml) or both, as appropriate.

2.2. Construction of mutants and reporter strains

Four oligonucleotide primers (see Table 2) were designed for each of the exonuclease loci to permit the 5′-end of the target gene to be amplified by one pair of primers and the 3′-end to be amplified by another set. The outer primer of each pair carried either a BamHI site or an EcoRI site at the 5′-end and the inner primers had a HindIII site at the 5′-end. The PCR products were cloned into pCR2.1TOPO (Invitrogen) and then released by digestion with either BamHI and HindIII or EcoRI and HindIII. These fragments for each gene were then used in three-way ligations with appropriately linearised pUC18ΔHindIII (i.e.

Table 1
Mutant strains and PV reporter constructs

Mutated gene(s)	Strain/reporter gene[a]			
	Rd/none	Rd/AGTC38R	Rd/AGTC17R	Rd/AT20R
exoI	RdΔ*exoI*	RdΔ*exoI*-38R	RdΔ*exoI*-17R	RdΔ*exoI*-AT20
recJ	RdΔ*recJ*	RdΔ*recJ*-38R	RdΔ*recJ*-17R	RdΔ*recJ*-AT20
recG	RdΔ*recG*	RdΔ*recG*-38R	RdΔ*recG*-17R	RdΔ*recG*-AT20
exoI/recJ	RdΔ*exoI*Δ*recJ* RdΔ*recJ*Δ*exoI*[b]	RdΔ*exoI*Δ*recJ*-38R		

[a] Reporter genes are *mod-lacZ* fusions containing 38 or 17 repeats of a tetranucleotide, 5′-AGTC, or 20 repeats of a dinucleotide, 5′-AT.
[b] Mutant RdΔ*exoI*Δ*recJ* contained tetracycline- and kanamycin-resistance cassettes in *exoI* and *recJ*, respectively. These cassettes were reversed in RdΔ*recJ*Δ*exoI*.