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P22 mediated recombination of *frt*-sites

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ABSTRACT

Flp mediated site specific recombination of *frt*-sites is frequently used in genetic engineering to excise, insert or invert DNA-cassettes in the chromosome. While constructs flanked by *frt*-sites are generally considered to be stable in the absence of the Flp enzyme, we observed that P22 chromosomes exceeding wild-type length tend to lose *frt*-flanked insertions via Flp independent recombination of *frt*-sites during phage propagation. This spontaneous recombination should be considered when engineering the chromosome of P22 and perhaps of other phages as well.

Introduction

Viruses of bacteria (bacteriophages or phages) are ubiquitous in the biosphere and display an extraordinary diversity (Rohwer, 2003; Breitbart and Rohwer, 2005; Clokie et al., 2011). So far, over 1000 complete bacteriophage genomes have been sequenced, revealing the existence of a plethora of phage-borne open reading frames of which the biological functions are yet to be elucidated (Hatfull and Hendrix, 2011). Many of these open reading frames likely code for proteins that are required for the proper conversion of host physiology during lytic infection, while others might support a more symbiotic relationship with the host during lysogenic proliferation of temperate phages (Fineran et al., 2009; Mebrhatu et al., 2014). During the latter, the phage chromosome is typically inserted into that of the host, after which the resulting prophage is synchronously replicated with the host chromosome. Yet other open reading frames might be dedicated to other phage–host interactions, as has recently been demonstrated for the *pid* moron gene of the temperate lambdoid P22 phage of *Salmonella enterica*, which seems to become expressed only during pseudo-lysogenic development (Cenens et al., 2013).

Functionally characterizing the many novel phage genes that are currently being discovered will benefit our understanding of phage biology and ecology, as well as fuel biotechnological applications of phages and their derivatives (Haq et al., 2012; Drulis-Kawa et al., 2012). While knock-out analysis would be an important tool in this context, the genetic manipulation of (pro)phages typically tends to be hampered by the density of genes in phage genomes and the maximum length of phage chromosome that can be properly packaged in the capsid, which increase the risk on polar effects and limit the number of extra bases that can be inserted, respectively. In this communication, we describe the observed side-effects of using *frt*-flanked antibiotic resistance cassettes during genetic engineering in phage P22.

Results

In a previous study (Cenens et al., 2013) Lambda-Red mediated recombination (Datsenko and Wanner, 2000) was used to replace the *pid* open reading frame with a *yfp-frt-cat-frt* cassette (with the *cat* gene conferring chloramphenicol resistance) in the P22 prophage chromosome of the corresponding *Salmonella* Typhimurium LT2 lysogen (resulting in P22Δ*pid*::*yfp-frt-cat-frt*). When this prophage was prepared from a late stationary phase culture and plated on a lawn of susceptible *S.* Typhimurium LT2 cells, however, turbid plaques were recovered that contained both chloramphenicol resistant (Cm^R) and chloramphenicol sensitive (Cm^S) lysogens. Subsequent PCR amplification and sequencing revealed that the Cm^R clones contained the P22Δ*pid*::*yfp-frt-cat-frt* prophage, while the Cm^S clones contained the P22Δ*pid*::*yfp-frt* prophage in which the *frt*-sites were perfectly recombined. Interestingly, this phenomenon could independently be confirmed in a P22 mutant harboring a *frt-nptII-frt* insertion (with the *nptII* gene conferring kanamycin resistance) in the superinfection exclusion gene *sieB*, indicating that

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Flp-independent recombination of *frt*-sites can occur on other sites of the P22 chromosome as well, and independently of the marker flanked by the *frt*-sites.

A more quantitative analysis revealed that, upon inoculation in lysogeny broth supplemented with chloramphenicol, 48 out of the 50 P22Δ*pid*::*yfp-frt-cat-frt* derived plaques examined (i.e. 96%) gave rise to Cm^R growth (indicative for the presence of Cm^R lysogens in the plaque). Nevertheless, when individual lysogens originating from a single plaque were examined, as much as 84% (59/70) proved to be Cm^S. Together these findings suggest (i) that upon prophage induction, most (if not all) of the produced virions harbored P22 chromosomes that still contained the (unflipped) *frt-cat-frt* cassette (otherwise Cm^R lysogens could never have been detected in the plaques that were subsequently produced), but (ii) that somehow during propagation within the developing plaque the *frt-cat-frt* cassettes became flipped, leading to the emergence of Cm^S lysogens.

To examine whether this site-specific recombination of *frt*-sites (in the absence of Flp) was the result of RecA activity of the *S.* Typhimurium LT2 host, the latter experiment was repeated in a *recA* host. More specifically, the fraction of Cm^S lysogens originating either from a single plaque or from different plaques was examined on an LT2, LT2 *srl*::*Tn10* and LT2 *recA1 srl*::*Tn10* host, yielding 83–92%, 87–89%, and 98–99% Cm^S lysogens respectively (Table 1), suggesting that the host's RecA function is not a prerequisite for *frt* recombination during P22Δ*pid*::*yfp-frt-cat-frt* propagation.

Since phage P22 relies on terminal redundant repeats ($1{,}600 \pm 750$ bp (Casjens and Hayden, 1988)) to circularize its linearly packed chromosome immediately after infection, and since the length of the P22Δ*pid*::*yfp-frt-cat-frt* chromosome exceeds that of the wild-type phage with 1,917 bp, we wondered whether reduced or abolished terminal redundancy could attenuate the fitness of P22Δ*pid*::*yfp-frt-cat-frt* chromosomes and hence the frequency of Cm^R lysogens. The latter hypothesis was tested by replacing the complete (non-essential) *gtrABC* operon with a *frt-nptII-frt* cassette, resulting in P22Δ*gtrABC*::*frt-nptII-frt* (Cenens et al., in preparation), thereby reducing chromosome size by 1,430 bp compared to wild-type P22. Interestingly, using the same experimental set up as described above, no *frt-nptII-frt* recombination could be scored within >500 lysogens originating from different P22Δ*gtrABC*::*frt-nptII-frt* plaques, indicating that the tendency to recombine *frt*-sites is abolished upon shortening the P22 chromosome.

Discussion

Based on our observations, the chromosome of phage P22 (and perhaps other phages as well) seems prone to incur recombination of engineered *frt*-sites (and perhaps of other short homologous repeats as well) when its length exceeds that of wild-type P22. In fact, the corresponding reduction or loss of terminal redundant repeats (naturally varying between 850 bp and 2,350 bp (Casjens and Hayden, 1988), and becoming reduced with the size of any insertion) during headful packaging of larger-than-wild-type P22 chromosomes likely compromises their subsequent circularization upon infection, and could explain the competitive advantage of the *frt*-recombination event.

Since this phenomenon seems not to require functional RecA of the *S.* Typhimurium host, a P22-borne factor might be responsible for this event. In this context, the P22 Erf (essential recombination factor) protein could be a candidate to perform this recombination, since it naturally serves to recombine the terminal redundant repeats of the infecting P22 chromosome prior to further phage propagation or lysogenization (Botstein and Matz, 1970; Susskind and Botstein, 1978; Benson and Roth, 1997). In fact, Erf has previously been shown to be responsible for recombining large duplications in plasmids upon their transduction with P22 (Garzón et al., 1995; Garzón et al., 1998). If involvement of Erf could be confirmed, it would imply that this factor is able to recombine even very short stretches of DNA, such as the 34 bp *frt*-sites.

Possible *frt*-instability should be taken into account during recombineering efforts in P22, and perhaps in other phages whose proliferation depends on headful packaging and terminal redundant repeats as well. While it can either interfere with experiments where an *frt*-flanked cassette needs to be maintained in the phage chromosome, this phenomenon could also be exploited as it mitigates the need for the Flp site specific recombinase to recombine *frt*-sites.

Table 1
Recombination of *frt*-sites in P22Δ*pid*::*yfp-frt-cat-frt* derived plaques on different hosts.

Host	Single plaque[a]		Different plaques[b]	
	#Cm^S lys./#lys.	% of Cm^S	#Cm^S lys./#lys.	% of Cm^S
LT2	40/48	83	82/89	92
LT2 *srl*::*Tn10*	63/72	87	80/90	89
LT2 *recA1 srl*::*Tn10*	70/71	99	94/96	98

[a,b]The results from two independent experiments are shown: either all lysogens came from a single plaque (a) or from 15 different plaques (b).
#lys = number of lysogens.

Materials and methods

Strains and growth conditions

S. Typhimurium LT2 wild-type, and its *srl::Tn10*, and *recA1 srl::Tn10* derivatives (Mebrhatu et al., 2011), as well as phage P22Δ*pid*::*yfp-frt-cat-frt* (Cenens et al., 2013) and P22Δ*gtrABC*::*frt-nptII-frt* (Cenens et al., in preparation) were used in this study. For culturing bacteria, lysogeny broth (LB) (Sambrook and Russell, 2001) was used. For agar plates, LB was supplemented with 1.5% (for spreading plates) or 0.7% (for soft-agar plates) agar. Cultures were grown in LB broth for 15–20 h at 37 °C under well-aerated conditions (200 rpm on an orbital shaker) to reach stationary phase, when appropriate, chloramphenicol (30 μg/ml final concentration) or kanamycin (50 μg/ml final concentration) was added to the growth medium.

Phage manipulations

Indicated P22 mutants were propagated on *S.* Typhimurium LT2 or its derivatives either as plaques in LB soft-agar or as lysates in LB as described previously (Davis et al., 1980). Phage stocks were filter sterilized with 0.2 μm filters (Fisher Scientific, Aalst, Belgium) and chloroform was added to maintain sterility.

To check for the presence of chloramphenicol resistant lysogens within plaques, plaques were plugged from the soft-agar and placed in LB supplemented with chloramphenicol, after which this culture was scored for growth. For gathering lysogenized clones, the middle of a plaque was sampled and streaked on EBU-plates (Maloy et al., 1996), which are used to discriminate phage infected from uninfected colonies. Non-infected colonies were picked and cross-streaked across P22 wild-type on fresh EBU-plates, with resistance to P22 infection being indicative of their lysogenic status. Please note in this context that the frequency of P22-resistant but non-lysogenic clones was below 10^{-2}.

Sequencing

Sequencings were performed by Macrogen Europe (Amsterdam, The Netherlands). Primers Pid_Fw (5′-ACAGGTCTAACGCTTCCC-3′) and Pid_Rev (5′-GACATCGGTTATTGCAGAGG-3′) were used to amplify and sequence the *frt* region in P22Δ*pid*::*yfp-frt-cat-frt* and P22Δ*pid*::*yfp-frt*.

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