

Prevalence, conservation and functional analysis of *Yersinia* and *Escherichia* CRISPR regions in clinical *Pseudomonas aeruginosa* isolates

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Here, we report the characterization of 122 *Pseudomonas aeruginosa* clinical isolates from three distinct geographical locations: Dartmouth Hitchcock Medical Center in New Hampshire, USA, the Charles T. Campbell Eye Microbiology Lab at the University of Pittsburgh Medical Center, USA, and the Aravind Eye Hospital in Madurai, India. We identified and located clustered regularly interspaced short palindromic repeats (CRISPR) in 45/122 clinical isolates and sequenced these CRISPR, finding that *Yersinia* subtype CRISPR regions (33 %) were more prevalent than the *Escherichia* CRISPR region subtype (6 %) in these *P. aeruginosa* clinical isolates. Further, we observed 132 unique spacers from these 45 CRISPR that are 100 % identical to prophages or sequenced temperate bacteriophage capable of becoming prophages. Most intriguingly, all of these 132 viral spacers matched to temperate bacteriophage/prophages capable of inserting into the host chromosome, but not to extrachromosomally replicating lytic *P. aeruginosa* bacteriophage. We next assessed the ability of the more prevalent *Yersinia* subtype CRISPR regions to mediate resistance to bacteriophage infection or lysogeny by deleting the entire CRISPR region from sequenced strain UCBPP-PA14 and six clinical isolates. We found no change in CRISPR-mediated resistance to bacteriophage infection or lysogeny rate even for CRISPR with spacers 100 % identical to a region of the infecting bacteriophage. Lastly, to show these CRISPR and *cas* genes were expressed and functional, we demonstrated production of small CRISPR RNAs. This work provides both the first examination to our knowledge of CRISPR regions within clinical *P. aeruginosa* isolates and a collection of defined CRISPR-positive and -negative strains for further CRISPR and *cas* gene studies.

Received 24 September 2010

Revised 11 November 2010

Accepted 12 November 2010

INTRODUCTION

The ability of mobile genetic elements to alter the fitness and/or phenotype of bacteria has been well established (Brüssow *et al.*, 2004). Recent studies in members of the genera *Pseudomonas*, *Streptococcus* and *Staphylococcus* have demonstrated the importance of clustered regularly interspaced short palindromic repeats (CRISPR) in the interaction of bacteria and mobile genetic elements such as plasmids and bacteriophage (Barrangou *et al.*, 2007; Marraffini & Sontheimer, 2008; Zegans *et al.*, 2009).

Abbreviations: CRISPR, clustered regularly interspaced short palindromic repeats; crRNA, CRISPR RNA; DHMC, Dartmouth Hitchcock Medical Center; EOP, efficiency of plaquing; PAM, proto-spacer-associated motifs.

A supplementary figure and five supplementary tables are available with the online version of this paper.

CRISPR are found in 39 and 88 % of sequenced bacterial and archaeal genomes, respectively (van der Oost *et al.*, 2009). Previous studies have shown that CRISPR regions have been categorized into eight distinct subtypes (*Escherichia*, *Yersinia*, *Neisseria*, *Desulfovibrio*, *Thermotoga*, *Haloarcula*, *Apenn* and *Mycobacterium*) that are widely distributed in bacteria and archaea (Haft *et al.*, 2005). CRISPR subtypes vary in both the sequence and length of their identical repeats as well as their repertoire of CRISPR associated (*cas*) genes (Haft *et al.*, 2005). Many CRISPR and their cognate *cas* genes are found in the same genomic location, termed a CRISPR region (Zegans *et al.*, 2009). So-called general *cas* genes are found in multiple CRISPR region subtypes (Haft *et al.*, 2005). Only one *cas* gene is found in all CRISPR region subtypes, namely the *cas1* gene (Haft *et al.*, 2005). However, each of these regions also harbours subtype-specific CRISPR-associated genes abbreviated *csx*,

where *x* is the first letter of the subtype (example: *csy* for the *Yersinia* subtype) (Haft *et al.*, 2005). Most *cas* genes are not shared between CRISPR region subtypes, calling into question the ability of different CRISPR regions to function similarly (Haft *et al.*, 2005).

Previous studies have implicated CRISPR in the resistance to lytic bacteriophage and plasmids (Barrangou *et al.*, 2007; Marraffini & Sontheimer, 2008). However, native resistance to bacteriophage and plasmids has only been observed in *Streptococcus thermophilus* and *Staphylococcus epidermidis*, respectively (Barrangou *et al.*, 2007; Brouns *et al.*, 2008; Marraffini & Sontheimer, 2008). While many bioinformatic studies have been published which match CRISPR spacer content to bacteriophage genomes (Díez-Villaseñor *et al.*, 2010; Held & Whitaker, 2009; Shah *et al.*, 2009; Vestergaard *et al.*, 2008), few studies have attempted to test the ability of these native CRISPR regions to mediate functional bacteriophage–host interactions.

Here, we report the prevalence, location and sequence of CRISPR regions from *Pseudomonas aeruginosa* clinical isolates, thus providing a library of *P. aeruginosa* spacers matching sequenced bacteriophages. We also report the host range and contribution to resistance of these CRISPR to sequenced *P. aeruginosa* bacteriophage. Finally, we show that there is no evidence supporting a role for the *Yersinia* subtype CRISPR in mediating native resistance to bacteriophage infection or lysogeny of *P. aeruginosa*.

METHODS

Bacterial and phage culture conditions. Bacterial strains, bacteriophage strains, plasmids and primers used in this study are listed in Supplementary Tables S1 and S2 (available with the online version of this paper). *P. aeruginosa* and *Escherichia coli* strains were routinely cultured in lysogeny broth (LB) at 37 °C (Bertani, 2004). The minimal medium M63 was supplemented with MgSO₄ (1 mM) and arginine (0.4 %).

Growth media were supplemented with antibiotics at the following concentrations: ampicillin (Ap), 150 µg ml⁻¹ (*E. coli*); gentamicin (Gm), 10 µg ml⁻¹ (*E. coli*) and 50 µg ml⁻¹ (*P. aeruginosa*); carbenicillin 50 µg ml⁻¹ (*E. coli*) and 500 µg ml⁻¹ (*P. aeruginosa*).

Deletion of *P. aeruginosa* clinical isolate *Yersinia* subtype CRISPR regions. Regions of sequence identity upstream and downstream of the UCBPP-PA14 CRISPR region were amplified and cloned into a pMQ30 vector, using *Saccharomyces cerevisiae* recombineering as previously performed (Shanks *et al.*, 2006). Clinical *P. aeruginosa* isolates harbouring *Yersinia* subtype CRISPR regions were spot mated with *E. coli* S17 harbouring the suicide pMQ30-ΔCR plasmid. Transformants were selected on gentamicin and nalidixic acid. Meridiploids were counter-selected on 5 % sucrose before being tested for the clean deletion of the entire CRISPR region by PCR. Deletions were confirmed with primers *csy3* amp For, *csy3* amp Rev, ΔCR Check For(70) and ΔCR Check Rev(70). ΔCR Check For(70) and ΔCR Check Rev(70) are located external to the regions of DNA sequence homology found on plasmid pMQ30-ΔCR (Supplementary Table S2). Primers *csy3* amp For and *csy3* amp Rev were used to amplify *csy3*, providing both a positive and negative control for each strain (Supplementary Table S2).

Northern blot analysis. Northern blot assays were performed to determine the ability of *P. aeruginosa* strains to functionally produce small CRISPR RNAs (crRNAs) under laboratory conditions. Cultures grown overnight in LB medium were pelleted and resuspended in 100 µl 400 µg lysozyme ml⁻¹ in TE buffer for 3–5 min at room temperature. Following lysozyme-mediated cell lysis, total RNA was purified using the *mirVana* miRNA isolation kit (Ambion), essentially following the manufacturer's instructions for total RNA extraction. Primers Northern Marker/probe For and Northern Marker/probe Rev2 were utilized in PCRs to synthesize a probe comprising the last four spacers and three repeats of CRISPR2. The random primed DNA labelling kit (Roche) and [α -³²P]dCTP [3000 Ci mmol⁻¹ (111 TBq mmol⁻¹); PerkinElmer] were utilized according to the manufacturer's specification to radioactively label the probe. *mirVana*-suggested sRNA prehybridization, hybridization and wash solutions were utilized at 65, 37 and 37 °C, respectively. These conditions were chosen because they allowed our probe, containing four CRISPR spacers and three CRISPR repeats, to hybridize with the conserved repeat sequence of all *Yersinia* subtype CRISPR-derived small crRNAs regardless of spacer content. Following the third 15 min wash, membranes were covered with plastic wrap and exposed to a storage phosphor screen (GE Healthcare) overnight. After exposure, phosphor screens were analysed on a Storm 860 (Molecular Devices) and processed using Image Quant software v5.2 (Molecular Devices).

Measurement of efficiency of plaquing (EOP). Bacteriophage lysates were prepared by filter-sterilizing overnight LB-grown cultures of either *P. aeruginosa* strains UCBPP-PA14 or PAO1 lysogenized by the given temperate bacteriophage. Top agar lawns of CRISPR-region-positive and -negative strains were prepared as described previously (Zegans *et al.*, 2009), and spotted with serial dilutions of three independent bacteriophage preparations. The EOP was determined by dividing the titre of a single bacteriophage preparation on the CRISPR region deletion strain by the titre obtained on the cognate CRISPR-region-positive strain. Standard deviations were obtained by comparison of the EOP obtained for three independent bacteriophage preparations.

Measurement of lysogeny frequency. Similar to EOP measurements, bacteriophage lysates were prepared by filter-sterilizing overnight LB-grown cultures of *P. aeruginosa* strain UCBPP-PA14 lysogenized by either DMS3 or MP22. Microcentrifuge tubes containing 500 µl of sterile LB medium were inoculated with 10 µl of an overnight culture of either wild-type *P. aeruginosa* UCBPP-PA14 or the ΔCR mutant. This dilute bacterial culture was then inoculated with 10 µl purified bacteriophage (10⁷ p.f.u. ml⁻¹) and incubated at 37 °C for 25 min. Following incubation, cultures were washed twice with fresh LB medium to remove all remaining infectious bacteriophage particles. Serial dilutions were performed, plated on LB agar and incubated overnight at 37 °C. Cross-streak test plates were prepared by dripping 30 µl bacteriophage lysate down the centre of an LB agar plate (Davis *et al.*, 1980). Single colonies were picked from plates and streaked perpendicularly across the central bacteriophage lysate line of the cross-streak test plates. Bacteria that had become lysogens were immune to super-infection by the same bacteriophage; however, non-infected bacteria grew poorly after passing through the concentrated bacteriophage lysate (Davis *et al.*, 1980). Three independent assays were performed for each strain, with 50 colonies cross-streaked for every experiment (150 total colonies per strain). Statistical analysis was performed using the Fischer exact test.

CRISPR region sequencing and annotation. Clinical isolates harbouring *Yersinia* subtype CRISPR regions were amplified by PCR with primers *Yersinia* subtype CR amp For(70) and *Yersinia* subtype CR amp Rev(70) using Phusion polymerase (New England Biolabs, Beverly, MA). The resulting ~12 kb product was gel purified

(Qiagen), denatured at 96 °C for 5 min, and sequenced on a ABI 3730 DNA analyser sequencer using primers *Yersinia* subtype CRISPR1 SEQ FOR(70), *Yersinia* subtype CRISPR1 SEQ REV(70), *Yersinia* subtype CRISPR2 SEQ FOR(70), and *Yersinia* subtype CRISPR2 SEQ REV(70). Sequencing gaps were closed using unique primers proximal or distal to the sequencing gap (primer sequences not shown). Annotation of each CRISPR1 and 2 was performed manually and entered into the redundant spacer library (Supplementary Table S3, available with the online version of this paper).

Clinical isolates harbouring *Escherichia* subtype CRISPR regions were treated similarly except primers *Escherichia* subtype CR amp For(72) and *Escherichia* subtype CR amp Rev(72) were used to amplify the ~10 kb region and primers *Escherichia* subtype CRISPR1 SEQ FOR(72), *Escherichia* subtype CRISPR1 SEQ REV(69), *Escherichia* subtype CRISPR2 SEQ FOR(70), and *Escherichia* subtype CRISPR2 SEQ REV(72) were utilized in sequencing reactions.

Redundant and non-redundant spacer libraries. The spacer content of each strain was entered into a redundant spacer library (Supplementary Table S3). We also created a non-redundant library in which each unique spacer was represented in the database only once (Supplementary Table S4, available with the online version of this paper). Each non-redundant spacer was then compared against the NCBI database using BLASTN to identify all 100 % identical spacer targets using the program iSpacer ([identify spacer](#)).

iSpacer. iSpacer was created to increase the speed and accuracy at which large spacer libraries can be compared to the NCBI sequence database. iSpacer is written in the Python programming language, and functions on both PC and Mac platforms. Spacer libraries in FASTA format are opened in iSpacer, compared against the entire NCBI database at various stringency levels, and the resulting proto-spacer matches are exported into .txt and Microsoft Excel formatted files. The iSpacer program and supporting material is available free upon request from the corresponding author.

RESULTS

Prevalence and location of *Pseudomonas aeruginosa* CRISPR regions

We determined the prevalence of *Yersinia* and *Escherichia* subtype CRISPR regions in 122 clinical isolates of *P. aeruginosa* from three distinct geographical locations. Fifty isolates were obtained from Dartmouth Hitchcock Medical Center (DHMC) (Lebanon, NH, USA), 43 were from the Charles T. Campbell Eye Microbiology Lab (Pittsburgh, PA, USA), and 29 were from Aravind Eye Hospital (Madurai, Tamil Nadu, India). Isolates were obtained from *P. aeruginosa* sputum, urine, cystic fibrosis, and eye infections. Given the geographical and clinical diversity of this collection, it was likely these isolates have experienced a wide range of CRISPR–bacteriophage interactions.

To determine the prevalence of *Yersinia* and *Escherichia* subtype CRISPR regions, we amplified the subtype-specific *cas* genes *csy1* and *cse3*, respectively. Primers specific for *csy1* and *cse3* were verified on the only two sequenced *P. aeruginosa* strains harbouring CRISPR regions (UCBPP-PA14 and PA2192). The conserved non-CRISPR/*cas* related gene *pilG* was utilized as a positive control for all

PCRs. Utilizing primers specific to *P. aeruginosa* strain UCBPP-PA14 *csy1* gene, we found that 40 of the 122 clinical isolates putatively harbour *Yersinia* subtype CRISPR regions (33 %). Campbell Eye Microbiology Lab samples had a slightly higher *Yersinia* subtype prevalence (42 %) than those from DHMC or Aravind Eye Hospital (22 and 38 %, respectively). Initially we believed that these CRISPR/*cas* regions would be randomly distributed in different locations throughout the clinical isolate genomes because CRISPR regions are thought to be highly mobile genomic regions (Chakraborty *et al.*, 2010). However, all 40 *Yersinia* subtype CRISPR regions we mapped were located between homologues of the PA14_33290 and PA14_33370 ORFs.

Likewise, primers specific to the *P. aeruginosa* strain PA2192 *cse3* gene led to the discovery of seven isolates harbouring *Escherichia* subtype regions (6 %). *Escherichia* subtype CRISPR regions were found in 10 % of Aravind Eye Hospital isolates, while only 6 and 2 % of DHMC and Campbell Eye Microbiology Lab samples, respectively, harboured these regions. Similar to the *Yersinia* subtype, all *Escherichia* CRISPR regions were found in the same genomic location, between the PA14_48160 and PA14_48150 ORF.

Interestingly, sequenced *P. aeruginosa* strain PA2192 and three of the seven clinical isolates harbouring an *Escherichia* subtype CRISPR region also contain a *Yersinia* subtype region.

While we believe that this PCR-based analysis of these clinical isolates is thorough, we likely overlooked strongly degenerate *Yersinia* and *Escherichia* subtype CRISPR regions, as well as CRISPR regions of other subtypes.

CRISPR region conservation and spacer sequence

Sequencing of CRISPR regions was accomplished by amplification of the entire ~12 kb *Yersinia* and ~10 kb *Escherichia* CRISPR regions by PCR (Fig. 1a), followed by gel purification and sequencing of PCR products. Initially, both *cas* genes and CRISPR were sequenced, but it was quickly evident that *cas* sequence and organization were well conserved with previously sequenced *P. aeruginosa* strains UCBPP-PA14 and PA2192. Thus, the high conservation of *cas* gene sequences prompted us to focus on sequencing the more variable CRISPR sequences. Intriguingly, both *Yersinia* and *Escherichia* CRISPR regions have similar organizations. Both regions harbour two CRISPR with slightly different repeat sequences flanking a series of *cas* genes (Fig. 1b).

To analyse the spacer content of these strains, we sequenced each CRISPR and created a redundant spacer library with every spacer from all of the CRISPR-positive clinical isolates analysed (Supplementary Table S3). In order to follow the standard numbering system for CRISPR spacers presented in Supplementary Table S3, we made the assumption based

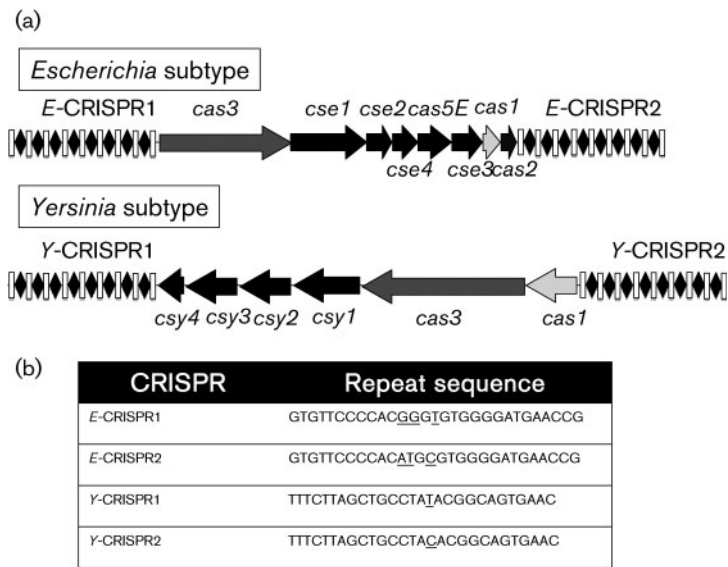


Fig. 1. Organization and conservation of *P. aeruginosa* CRISPR regions. (a) Organization of *cas* genes and flanking CRISPR are shown for the *Escherichia* subtype (top) and *Yersinia* subtype (bottom). Spacers are shown as diamonds and repeats as rectangles. Arrows indicate *cas* genes and their predicted direction of transcription. (b) Repeat sequences of the *Yersinia* and *Escherichia* subtypes (differences are underlined).

on published studies (Barrangou *et al.*, 2007; Deveau *et al.*, 2008) that spacer acquisition is polar, taking place at one end of a given stretch of each CRISPR. Convention states that spacers are numbered starting from the terminus where new spacers are acquired (Deveau *et al.*, 2008). The data used to support the numbering system presented in Supplementary Table S3 are presented in Supplementary Fig. S1 (available with the online version of this paper). It is worth noting that the most prevalent spacer repertoires for *Yersinia* and *Escherichia* subtype CRISPR are similar to the CRISPR spacer sequences found in *P. aeruginosa* strains UCBPP-PA14 and 2192, respectively.

We also created a non-redundant library of all sequenced spacers, which is presented in Supplementary Table S4. This non-redundant spacer library contains 656 unique spacers, which were independently compared against the NCBI database. This comparison was performed using the newly developed iSpacer program, which can compare large lists of spacer sequences to the NCBI database using BLASTN, to determine regions of the bacterial and bacteriophage genomes that are identical to spacer sequences.

The sequences identified by using iSpacer analysis could be categorized into five groups, those spacers matching: (i) sequenced lysogenic *P. aeruginosa* bacteriophage ($n=65$), (ii) prophages within sequenced *P. aeruginosa* clinical isolates ($n=95$), (iii) transposons ($n=2$), (iv) pathogenicity islands ($n=21$), and (v) previously sequenced *P. aeruginosa* CRISPR regions (Supplementary Table S4).

Among the spacers that showed 100% identity to non-CRISPR sequences, 65 independent spacers were 100% identical to portions of 12 sequenced, well-characterized lysogenic *P. aeruginosa* bacteriophage. Fig. 2 illustrates the location of spacers matching eight representative sequenced bacteriophages: DMS3, MP22, D3112, D3, PAJU2, B3, Φ CTX and F116 (Braid *et al.*, 2004; Byrne &

Kropinski, 2005; Heo *et al.*, 2007; Kropinski, 2000; Nakayama *et al.*, 1999; Uchiyama *et al.*, 2009; Wang *et al.*, 2004; Zegans *et al.*, 2009). Further, 95 unique spacers were found to match uncharacterized prophages on the genomes of *P. aeruginosa* clinical isolates. These prophages were sequenced as part of bacterial genome sequencing projects or obtained from metagenomic studies of cystic fibrosis isolates (Hayden *et al.*, 2008; Roy *et al.*, 2010; Winstanley *et al.*, 2009). Interestingly, a number of these spacers matched sequences of prophages linked to the increased virulence of the Liverpool epidemic strain LESB58 (Supplementary Table S5) (Winstanley *et al.*, 2009). Only two spacers matched to transposons; oddly these spacers were also the only spacers that matched to non-*P. aeruginosa* sequences (these non-transposon matches were not included in Supplementary Table S4). Two spacers were also identical to pyocin gene fragments, which are vestigial bacteriophage tail fibres used by *P. aeruginosa* to puncture the membrane of competing organisms (Lee *et al.*, 1999).

We also identified 21 unique spacers that matched to *P. aeruginosa* genomic islands (PAGI). Great care was taken to differentiate spacer matches within putative lysogenic bacteriophage genomes from those matching PAGI. Twelve unique spacers were 100% homologous to PAGI-6, of which five also matched to the Φ CTX genome, a finding consistent with previous reports detailing the sequence conservation between the mobile genomic island PAGI-6 and the cytotoxin-encoding bacteriophage Φ CTX (Battle *et al.*, 2009). PAGI-5 and the *P. aeruginosa* UCBPP-PA14 pathogenicity island 1 (PAPI-1) are members of a large family of genomic islands closely related to large integrating 103.5 kb pKLC102-like plasmids first discovered in *P. aeruginosa* clone C strains (Battle *et al.*, 2009). Nine unique spacers matched to PAGI-5-like elements, such as PAPI-1.

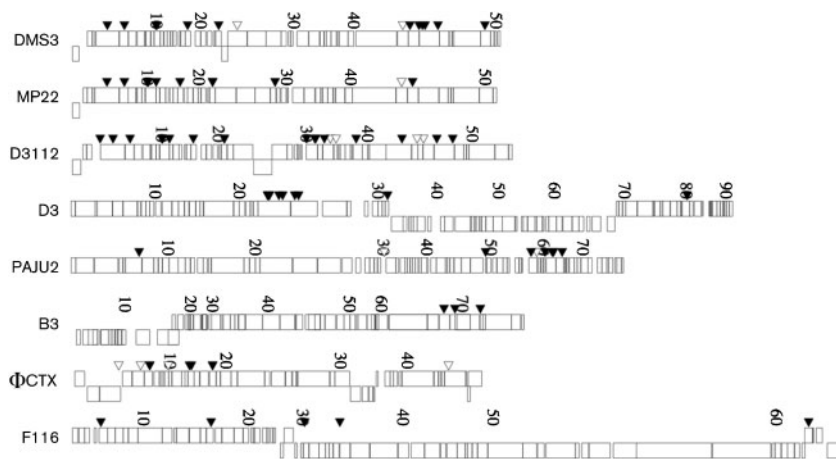


Fig. 2. Location of spacers 100% identical to common temperate *P. aeruginosa* bacteriophage. Boxes denote known or putative bacteriophage ORFs that have been published previously. Forward oriented ORFs are depicted above those ORFs that are divergently transcribed. Each arrow signifies the site of a proto-spacer sequence 100% identical to a spacer identified in our analysis, with *Yersinia* subtype spacer matches shown as black triangles and *Escherichia* subtype spacer matches shown as open triangles.

Proto-spacer matches and proto-spacer-associated motifs (PAMs)

We analysed the flanking sequence of the putative proto-spacers corresponding to the unique spacers identified here (data not shown) and found conserved PAMs that were different between *Yersinia* and *Escherichia* subtypes. *Yersinia* subtypes contained a conserved GG dinucleotide immediately adjacent to their spacer, while *Escherichia* subtypes had a consensus CTT PAM. Both of these PAM motifs matches those previously discovered using spacer matches from many bacterial species containing the same CRISPR subtype (Mojica *et al.*, 2009). Our large library of 100% identical spacer matches also showed that PAGI, prophages and previously sequenced bacteriophage all retained the same PAM sequence for both CRISPR subtypes (data not shown).

Analysis of CRISPR-mediated resistance using EOP and lysogeny assays

Previous studies implicated CRISPR and *cas* genes in resistance to lytic bacteriophages and to plasmids (Barrangou *et al.*, 2007; Marraffini & Sontheimer, 2008). To test the ability of the more prevalent *Yersinia* subtype CRISPR regions to mediate bacteriophage resistance, we identified clinical isolates with CRISPR regions harbouring one or more unique spacers 100% identical to sequenced bacteriophage. We then created mutant strains lacking the entire CRISPR region, and these deletion strains served as a control for non-CRISPR-mediated interactions. We assessed the resistance of each wild-type clinical isolate and its CRISPR deletion derivative to bacteriophage infection using an assay that measured the EOP.

Sequenced bacteriophage D3, DMS3, MP22 and F116 were utilized to infect clinical isolates harbouring spacers 100% identical to proto-spacer regions within the given bacteriophage. For most bacteriophages tested, we observed no ability to plaque on the clinical strains tested regardless of the presence or absence of the CRISPR region (Table 1). For those strains that could support plaquing by DMS3

and/or MP22, we found no evidence for CRISPR-dependent resistance to bacteriophage as judged by a plaque assay (Table 1). That is, the EOP was approximately 1 in all experiments. These data indicate that for the strains and bacteriophage tested, CRISPR did not confer resistance to infection.

To determine if *Yersinia* subtype CRISPR regions harbouring 100% identical spacer matches to temperate bacteriophage impact the rate at which lysogeny occurs, we tested the frequency at which *P. aeruginosa* UCBPP-PA14 was lysogenized by bacteriophages MP22 and DMS3 in the presence or absence of the *Yersinia* subtype CRISPR region (harbouring one or more spacers 100% identical to these bacteriophage). We observed, by cross-streak test, that 8% of CRISPR-positive bacteria and 6% of Δ CR bacteria became lysogenized by bacteriophage DMS3. Similarly, 2% of CRISPR-positive and 1.3% of Δ CR bacteria were lysogenized by the MP22 bacteriophage. Both of these lysogeny rates were not found to be significantly different

Table 1. Measuring EOP of clinical isolates

EOP is given as mean \pm SD. EOP of 1 indicates no change in resistance. NCR, Non-CRISPR-mediated resistance, assessed by the lack of plaque formation in the CRISPR-negative clinical isolate.

Strain	Bacteriophage	EOP
PA14 Δ CR	DMS3	1.06 \pm 0.2
	MP22	1.95 \pm 1.0
	F116	NCR
SMC4485 Δ CR	DMS3	1.53 \pm 0.5
	MP22	2.10 \pm 1.6
	F116	NCR
SMC4515 Δ CR	MP22	0.92 \pm 0.3
	F116	NCR
SMC4513 Δ CR	DMS3	NCR
SMC4384 Δ CR	D3	NCR
SMC4502 Δ CR	DMS3	NCR
SMC4504 Δ CR	F116	NCR

between CRISPR-positive and -negative strains using the Fisher exact test ($P=0.65$ and $P=1$, respectively).

crRNA production from clinical isolates

We next assessed whether the CRISPR transcript in the clinical isolates described above were processed into small RNAs. From the presence of crRNA we could infer that the *cas* genes were expressed and each product(s) was functional (Haurwitz *et al.*, 2010). Northern blot analysis showed that each strain produced crRNAs, and the production of these crRNAs was dependent on the presence of the CRISPR region (Fig. 3). These data indicate that *Yersinia* subtype *P. aeruginosa* CRISPR regions are expressed and functionally able to produce crRNAs under our experimental conditions.

DISCUSSION

This work demonstrates that *P. aeruginosa* *Yersinia* subtype CRISPR did not mediate native resistance to bacteriophages, even when the bacteriophages contained sequences that were 100 % identical to spacers within the CRISPR. Widely accepted CRISPR dogma would suggest that when challenged by the bacteriophage with sequences 100 % identical to spacers, these CRISPR regions should respond by mediating resistance to the given bacteriophage (Barrangou *et al.*, 2007). To test this model with the *Yersinia* subtype CRISPR, we analysed multiple clinical isolates harbouring a diverse range of spacers against a collection of sequenced *P. aeruginosa* bacteriophage. However, we found that of the seven strains tested, none mediated CRISPR-dependent resistance to bacteriophage. Further, *P. aeruginosa* strain UCBPP-PA14, which harbours spacers 100 % identical to bacteriophage MP22 and DMS3, showed no difference in the rate of lysogeny for either of these temperate bacteriophage in the wild-type versus Δ CR strains. Thus, two of the previously described host-bacteriophage interactions mediated by CRISPR (Barrangou *et al.*, 2007; Edgar & Qimron, 2010) are not

impacted by the *Yersinia* subtype CRISPR region. The observation that *Yersinia* subtype CRISPR regions produced small crRNA in all of the strains tested demonstrates that these regions are functional under our laboratory conditions. Thus, our findings argue against the conclusion that these regions are 'silent', as has been suggested for CRISPR regions within *E. coli* (Díez-Villaseñor *et al.*, 2010; Pougach *et al.*, 2010; Westra *et al.*, 2010).

While previous studies of other CRISPR region subtypes have reported the importance of short PAM motifs adjacent to the bacteriophage genomic sequence matching to CRISPR region spacers (proto-spacers) (Deveau *et al.*, 2008), no study has demonstrated their requirement in *Yersinia* subtype CRISPR-mediated interactions. Our analysis of bacteriophage MP22 demonstrated no CRISPR-dependent alteration in EOP or frequency of lysogeny when the bacterial host strain harbours a spacer 100 % identical to a bacteriophage proto-spacer with a conserved PAM. Unfortunately, in this work we did not observe any CRISPR-dependent alteration in lysogeny rate or bacteriophage resistance, and so cannot comment on the requirement of the PAM motifs in our studies.

Our analysis showed that the *Yersinia* and *Escherichia* subtype CRISPR regions of *P. aeruginosa* clinical isolates harbour spacers against mobile genetic elements that integrate within the host chromosome. All 132 unique spacers 100 % identical to viral sequences match to either well-studied temperate *P. aeruginosa* bacteriophage capable of integrating into the host chromosome or prophages within the genome of *P. aeruginosa* clinical isolates (Braid *et al.*, 2004; Byrne & Kropinski, 2005; Heo *et al.*, 2007; Kropinski, 2000; Lee *et al.*, 1999; Nakayama *et al.*, 1999; Roy *et al.*, 2010; Uchiyama *et al.*, 2009; Wang *et al.*, 2004; Winstanley *et al.*, 2009; Zegans *et al.*, 2009). We also identified additional spacer sequences that match PAGI and transposons. Spacers matching to extrachromosomally replicating bacteriophage, which is typical of lytic bacteriophage, and plasmids are conspicuously absent from our dataset. Our data are also consistent with a previous report describing *Escherichia* and *Yersinia* subtype CRISPR regions which stated that of 100 *E. coli* strains tested, 65.4 % of bacteriophage-targeting spacers corresponded to a prophage within the *E. coli* isolates E24377A and SE11 (Díez-Villaseñor *et al.*, 2010). This seemingly preferential targeting of prophages coupled with spacers matching to PAGI and transposons raises the possibility that the natural role of these CRISPR regions is in the interaction with chromosomally integrated mobile genetic elements rather than in mediating defence against extra-chromosomal infecting bacteriophages and plasmids.

How different CRISPR region subtypes interact with proto-spacer targets is an area of active research and speculation. However, when one considers that *cas1*, which is thought to insert new spacers, is the only *cas* gene found in all CRISPR subtypes it is perhaps less surprising that these regions may have been co-opted for varied roles in

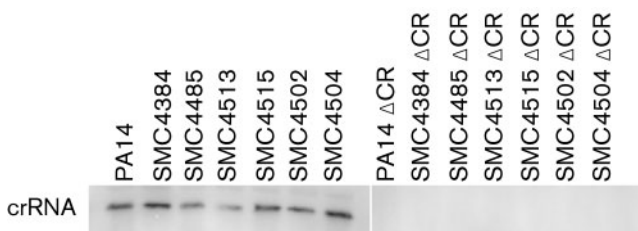


Fig. 3. Characterization of CRISPR-positive and -negative *P. aeruginosa* clinical isolates. Northern blot analysis of small crRNA production for the wild-type lab strain and six clinical isolates (left) and their Δ CRISPR derivatives (right). In this experiment, the three spacers and two repeats closest to the *cas1* gene of CRISPR2 of *P. aeruginosa* UCBPP-PA14 were used as the probe.

different bacteria. An example of this diversity of action is that the *Neisseria* subtype CRISPR region of *Streptococcus thermophilus* is believed to interact with the DNA of invading bacteriophage, while the *Thermotoga* subtype of *Pyrococcus furiosus* has been shown to interact directly with RNA (Hale *et al.*, 2009).

We found 41 unique clinical *P. aeruginosa* CRISPR spacers 100% identical to known horizontally transferable virulence factors, as well as 112 spacers identical to other putative virulence factors within PAGI, prophages and lysogenic bacteriophage. Transposons, prophages and genomic islands are thought to be responsible for the majority of strain diversity in *P. aeruginosa* (Battle *et al.*, 2008, 2009; Winstanley *et al.*, 2009). This type of genomic diversity is perhaps best exemplified by the Liverpool epidemic strain of *P. aeruginosa* (strain LESB58). Prior to this LESB58 study, it was assumed that most *P. aeruginosa* strains had very similar genomic content and few unique virulence factors that would make one strain considerably more virulent than another. However, unbiased signature-tagged mutagenesis coupled with genome sequencing revealed that many of the strain-specific genomic islands and prophages within strain LESB58 were critical to the increased virulence of this strain of *P. aeruginosa* (Winstanley *et al.*, 2009). Strikingly, three of the four prophage genomes implicated in contributing to LESB58 virulence in a chronic rat lung model of infection were targeted by one or more unique spacers within our library. Similarly, we identified many unique spacers targeting the previously identified virulence-modulating lysogenic *P. aeruginosa* bacteriophages D3 and Φ CTX. In addition, nine unique spacers targeted PAGI-5/PAPI-I-like elements, which have been shown to contribute to the virulence of *P. aeruginosa* clinical isolate PSE9 within the mouse model of acute pneumonia (Battle *et al.*, 2008). The observation that human clinical isolates of *P. aeruginosa* have acquired and retained spacers 100% identical to known and putative chromosomal virulence factors raises the intriguing hypothesis that these CRISPR and *cas* genes are mediating a clinically important interaction between the bacterial host and mobile genetic elements.

ACKNOWLEDGEMENTS

We thank Rory T. Allar, Dae Gon Ha and Thomas Hampton for preliminary experiments or statistical analysis. Bacteriophage or bacteria strains were contributed by the Cho lab (Sogang University, South Korea), Collection de phages Félix d'Herelle (Université Laval, France), the Maillard lab (Cardiff University, UK), the Lory Lab (Harvard Medical School, USA) and the Charles T. Campbell Eye Microbiology Lab (University of Pittsburgh, USA). This work was supported by a National Institutes of Health predoctoral fellowship to K. C. C. (T32 AI007519), the Dartmouth Dickey Center, and National Institutes of Health grants R01AI083256 and R21AI068662 to G. A. O.

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Edited by: S. D. Bentley