Monitoring genome evolution ex vivo: reversible chromosomal integration of a 106 kb plasmid at two tRNA\textsuperscript{Lys} gene loci in sequential \textit{Pseudomonas aeruginosa} airway isolates

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The genome rearrangements in sequential \textit{Pseudomonas aeruginosa} clone K isolates from the airways of a patient with cystic fibrosis were determined by an integrated approach of mapping, sequencing and bioinformatics. Restriction mapping uncovered an 8–9 kb deletion of PAO sequence between \textit{phnAB} and \textit{oprL} in clone K, and two 106 kb insertions either adjacent to this deletion or several hundred kilobases away, close to the \textit{pilA} locus. These 106 kb blocks of extra DNA also co-existed as the circular plasmid pKLK106 in several clone K isolates and were found to be closely related to plasmid pKLC102 in \textit{P. aeruginosa} clone C isolates. The breakpoints of the deletion in clone K and the \textit{attB–attP} sequences for the reversible integration of the plasmid in clones C and K were located within the 3’ end of the lysine tRNA structural genes (\textit{att} site). pKLK106 sequentially recombined with either of the two tRNA\textsuperscript{Lys} genes in clone K isolates. The \textit{att} site of the \textit{pilA} hypervariable region has been utilized by clone C to target its plasmid pKLC102 into the chromosome; the \textit{att} site of the \textit{phnAB–oprL} region has been employed by strain PAO to incorporate a DNA block encoding pyocin, transposases and IS elements. The use of typical phage attachment sites by conjugative genetic elements could be one of the major mechanisms used by \textit{P. aeruginosa} to generate the mosaic genome structure of blocks of species-, clone- and strain-specific DNA. The example described here demonstrates the potential impact of systematic genome analysis of sequential isolates from the same habitat on our understanding of the evolution of microbial genomes.

**Keywords:** cystic fibrosis, genome evolution, \textit{Pseudomonas aeruginosa}, recombination, tRNA gene

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### INTRODUCTION

The ubiquitous \textit{Pseudomonas aeruginosa} is endowed with remarkable genome diversity. Its genetic repertoire is made up of a 5–7 Mb large circular haploid chromosome, plasmid(s), (pro)phage(s) and other mobile genetic elements (de Bruijn \textit{et al}., 1998; Schmidt \textit{et al}., 1996). Although the contig of common genes is conserved in almost all \textit{P. aeruginosa} strains (Heuer \textit{et al}., 1998a) and its sequence diversity of about 0–3\% is one order of magnitude lower than in comparable housekeeping genes of enterobacteria (Kiewitz & Tümmler, 2000), 1–200 kb blocks of clone- or strain-specific DNA interrupt the conserved gene contig and give rise to a mosaic genome structure (Römling \textit{et al}., 1997). The 15–30\% portion of intra- and interclonal genomic diversity is not equally distributed, but clusters in hypervariable regions of the chromosome (Römling \textit{et al}., 1995; Heuer \textit{et al}., 1998a).

Current knowledge about genome diversity results mostly from comparative mapping and sequencing of selected isolates (Casjens, 1998) or propagation of bacterial populations in defined laboratory environments (Papadopoulos \textit{et al}., 1999). These approaches...
inherently provide only indirect evidence of how microbial genomes evolve in complex natural habitats. For the past 15 years we have collected P. aeruginosa airway isolates taken from patients with cystic fibrosis (CF) since the onset of colonization (Römling et al., 1994). The P. aeruginosa infections in CF are a paradigm of how versatile environmental bacteria can conquer, adapt and persist in an atypical habitat, and successfully evade defence mechanisms and chemotherapy in a susceptible host (Tümmler & Kiewitz, 1999). This strain collection provides a unique opportunity to monitor genome evolution. Here we report on the aetiology of genome rearrangements in sequential isolates from a patient with CF who has been and still is harbouring the most prevalent P. aeruginosa clones C and K (Römling et al., 1995). A large plasmid was found to integrate and mobilize repeatedly from an attB site located within two separate copies of a lysine tRNA gene. Both tRNA loci are located in hypervariable regions: one copy is located adjacent to pilA (Heuer et al., 1995).

**METHODS**

**Bacterial strains.** To monitor genomic variation of closely related P. aeruginosa strains, sequential airway isolates from a patient with CF were selected: K (clone SS3 in Heuer et al., 1998a, b), first P. aeruginosa-positive respiratory specimens; K1, K1’ (SS5, SS5’), 7 months; K2 (SS20), 36 months of colonization. For comparison, P. aeruginosa strain C, which belongs to a major clone in CF patients and aquatic habitats (Schmidt et al., 1996), and the reference strain PAO (DSM 1707) were included.

**Restriction mapping.** P. aeruginosa bacteria (5 × 10^9 P. aeruginosa cells ml^-1), grown to late exponential phase were encapsulated into agarose plugs, lysed extensively with detergents and proteinase K, and the intact chromosomes cleaved with SpeI or I-CeuI as described previously (Römling et al., 1997). In one-dimensional PFGE, the SpeI digests were separated in a Bio-Rad CHEF-DR III cell using three linear ramps of 5–60 s (17 h), 10–25 s (14 h) and 1–5 s (6 h) (E = 6 V cm^-1, 13 °C, 1% agarose gel with 10 μM thiorurea in 0.5 × TBE buffer).

For two-dimensional macrorestriction mapping (see Schmidt et al., 1996), the genome was completely digested with I-CeuI and separated in the first dimension in a Bio-Rad CHEF-DR III cell (E = 13 V cm^-1, one linear ramp of 400–1000 s in 1 s increments, 96 h, 13 °C, 0.6% agarose gel with 10 μM thiorurea). The entire lane of each strain was cut out, digested with SpeI and separated perpendicular to the first run in the second dimension using three linear ramps from 10–50 s (24 h), 10–25 s (22 h) and 1–10 s (14 h) (E = 6 V cm^-1, 13 °C, 1.5% agarose gel with 10 μM thiorurea).

For high-resolution Smith/Birnstiel mapping, SpeI complete/n partial double digestions (n: EcoRI, BglII, XhoI, NdeI) were carried out, the fragments separated by PFGE and hybridized with cloned SpeI fragment ends as described previously (Heuer et al., 1998b).

**Spel maps of strains K, K1 and K2 covering the toxA–hemA region were reconstructed by partial SpeI digestions of agarose-embedded chromosomes (1 × 10^10 P. aeruginosa cells ml^-1) using 0.025 U, 0.01 U or 0.005 U SpeI (New England Biolabs) for 1 h. The partial SpeI digests were separated in a Bio-Rad CHEF-DR III cell with three linear ramps of 5–100 s (30 h), 10–40 s (10 h) and 70–100 s (10 h) (E = 6 V cm^-1, 13 °C, 1.5% agarose gel with 10 μM thiorurea) and transferred onto nylon membranes by capillary blotting. Southern hybridizations were performed with PCR-amplified PAO sequences (http://www.pathogenesis.com/), cloned genes (Schmidt et al., 1996), cloned SpeI fragment ends (Heuer et al., 1998a) and SpeI linking clones (Römling & Tümler, 1993). Probes were labelled with digoxigenin-dUTP and hybridized with the pulsed-field blot as described previously (Schmidt et al., 1996). Hybridized fragments were detected by chemiluminescence using an alkaline phosphatase-conjugated anti-digoxigenin antibody and CDP-Star (Tropix) as substrate.

**Isolation and analysis of P. aeruginosa plasmids pKLC102 and pKLK106.** High-molecular-mass plasmids were prepared on a large scale by modified alkaline lysis. Plasmid size was determined by adding up the sizes of restriction fragments after conventional or PFGE gel separation of BamHI or SpeI digests, respectively. A library of partially BamHI-restricted pKLC102 was maintained as pLABR3-derived cosmids (Staskawicz et al., 1987) in Escherichia coli DH5α. For cosmid ordering, about 60 clones were digested with BamHI to completion and manually assembled by restriction fingerprint analysis. The fragment assembly was confirmed by Southern hybridization using single cosmids, single BamHI fragments (e.g. BmQ) or the small SpeI fragment SpAQ served as probes.

**PCR amplification and sequencing.** Chromosomal DNA was prepared using a rapid method for Gram-negative bacteria (Chen & Kuo, 1993). PCR was performed from purified DNA as described previously (Spangenberg et al., 1995). Primer sequences for PCR were designed to anneal to the PAO genome sequence between phnAB and oprL, the PAO sequence upstream of the pilA locus and the BamHI fragment sequence BmQ of pKLC102. The sequences are shown in Table 1. Selected PCR products were sequenced in both directions.

**Annotation.** The phnAB and the pilA regions of the PAO genome sequence (http://www.pathogenesis.com/) were searched for homologies in public databases with the pro-

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**Table 1. Primer sequences used in this study**

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Sequence data. Novel nucleotide sequences were deposited in GenBank. The accession numbers are AF285416–AF285426.

RESULTS

Two-dimensional PFGE and Southern analysis indicate genome rearrangements in sequential clone K isolates from a CF patient

Genome evolution of P. aeruginosa in CF lungs was monitored by analysis of sequential isolates following the onset of colonization. One patient has been and still is chronically co-colonized with the prevalent genotypes C and K (patient 11, Römling et al., 1994). Genotype K and C strains are 65% similar in their SpeI band patterns (Römling et al., 1995), indicating that these two genotypes are more closely related to each other than the average pair of P. aeruginosa genotypes, which share only 40% of bands in their SpeI fragment patterns (Grothues & Tümmler, 1991).

SpeI fragment patterns of patient 11’s clone K sequential isolates changed within the first 3 years of colonization (Fig. 1). The basis for these shifts of fragment size was not that these were nucleotide substitutions in SpeI recognition sites, but gross genome rearrangements, as became evident from two-dimensional PFGE of complete I-CeuI/SpeI digestions of clone K, K1 and K2 chromosomes (Fig. 2). The intron-encoded endonuclease I-CeuI cleaves within the four ribosomal operons of P. aeruginosa (Liu et al., 1993). The similarity of the SpeI fragment patterns reflects the conservation of most fragment sizes in the four I-CeuI fragments CeA, CeB, CeC and CeD, but a few SpeI fragments on CeA and CeD were unique for strains K, K1 and K2, and a 29 kb fragment was either located in CeA (K) or in CeD (K1 and K2) (Fig. 2). Two of these unique fragments (strain K: 46 kb; strains K1 and K2: 169 kb; Fig. 2) were found to hybridize with strain PAO fragment SpU. We knew from previous Southern hybridizations that the phnAB hypervariable region, which maps to SpU in strain PAO, was affected by the complex genome rearrangements in the sequential clone K isolates (Heuer et al., 1998a, b). The 1-5 Mb region flanking this hypervariable region was mapped by Southern hybridization of SpeI complete and partial digestes of strain K, K1 and K2 chromosomes with PAO-derived gene probes (Fig. 3). The global gene contig turned out to be conserved between PAO and the clone K strains as had been expected from the comparative two-dimensional analysis (see Fig. 2). The SpeI fragment contig and sizes were identical within experimental error for the adjacent four fragments towards the terminus of replication, but were divergent towards the origin of replication (Fig. 3).

An island of PAO sequence in the phnAB–oprL region is absent in clone K

As indicated by high-resolution restriction maps (Fig. 4), 95 % of the PAO SpU sequence was retained in clone K isolates, but approximately 5 % was deleted at the SpU/SpF junction. Taking the complete PAO genome sequence as the blueprint, oligonucleotides were designed to cover the terminal 10 kb of SpU by overlapping PCR products. Restricted clone K DNA hybridized only with those PCR products that had been amplified from PAO sequence with identical restriction fingerprints in PAO and clone K strains. These Southern data verified the absence of the terminal 7 kb of PAO SpU in clone K and narrowed the breakpoint of the conserved sequence contig to within 1 kb (Fig. 4). The breakpoint was located in a region that, according to our annotation, contains IS elements, a pyocin, integrase and orphan genes, but no housekeeping gene in strain PAO (Fig. 4).

Clones K and C harbour highly similar plasmids

Several clone K isolates harboured the episcopal 106 kb plasmid pKLK106. The plasmid contained a 29 kb SpeI fragment which co-migrated with, and was shown to
Fig. 2. Comparative analysis of *P. aeruginosa* strains PAO (DSM 1707), K, K1 and K2 by two-dimensional PFGE of sequential I-CeuI/SpeI restriction digests. After separation of the I-CeuI digested chromosomes in the first dimension, each lane of the four strains containing four I-CeuI fragments (CeA, CeB, CeC, CeD) was cut out, digested with SpeI and separated in the second dimension. In strains K, K1 and K2, a complete SpeI digest (lane termed ‘total’) is shown in the left lane. For better orientation, sizes of selected PAO SpeI fragments are indicated on the left as molecular mass standards. On the right, the differences between the three clone K strains are indicated. The 29 kb fragment co-migrates with invariant fragments of similar size as a double band either in the I-CeuI fragment CeA in strain K or in CeD in strains K1 and K2 and hence can be identified by signal intensity.

Fig. 3. Comparison of SpeI maps of *P. aeruginosa* PAO (DSM 1707), clonal variants K, K1 and K2, and strain C in the *toxA*–*hemA* region. SpeI fragment designations of PAO and C were taken from Schmidt et al. (1996). All fragment sizes (in kb) were determined by PFGE (refer to Fig. 1). The following probes were used in Southern hybridization analysis: single genes (*toxA*, *phnA*, *toxR*, *pilA* and *hemA*), cloned SpU fragment ends (3A1-2, 9H11), PAO-derived SpeI linking clones indicated by grey shaded boxes and *rrn* operons marked by black arrows. Corresponding map positions are connected by dotted lines. The grey highlighted areas indicate the integrated plasmids (pKLK106 in strains K, K1, K2 and pKLC102 in strain C). The PAO-specific SpU–SpF junction is shaded grey.
Fig. 4. High-resolution Smith/Birnstiel map (SpeI, EcoRI, BglII, XhoI, Ndel) of the hypervariable SpU–SpF junction in P. aeruginosa PAO and of the corresponding regions in clone K. The annotation of the PAO sequence is depicted as follows: established P. aeruginosa genes are black, putative ORFs showing highly significant homologies to known genes of the same or other species are light grey, genes located on IS elements are dark grey, toxin genes are black and white patterned, and orphan genes are white. The numbers refer to putative ORFs showing pronounced amino acid identities to (1) 3-oxoacyl-[acyl carrier protein]-synthase III, (2) acyl-CoA ligase precursor, (3) DNA methyltransferase, (4) type-1 fimbrial assembly protein, (7) pyocin S of P. aeruginosa, (10) regulatory protein ExsB of Synechocystis. The ORFs 5 (transposase) and 6 are located on IS1209, which belongs to the IS3 family and shows 87% nucleotide identity to IS1209 of P. aeruginosa (Burrows et al., 1996). ORFs 8 and 9 are part of the second copy of IS222 in the P. aeruginosa genome (Kropinski et al., 1994). The 76 bp tRNA\textsuperscript{Lys} gene and the att or att* elements (3’ terminal 45 or 21 bp of the tRNALys gene with a T overhang) are indicated by black bars. They flank the 8.9 kb insertion in PAO and the integrated 106 kb plasmid pKLK106 in strain K. The dotted line marks the integration point in strains K1 and K2. As evident from Figs 5 and 6a the plasmid recombination site is located on a 2–4 kb BamHI fragment (BmQ) forming the plasmid/chromosome junction in strain K. Triangles indicate oligonucleotide primers and asterisks depict matched pairs of complementary oligonucleotide primers.

BmQ was identical to the 3’ region of a tRNA\textsuperscript{Lys} gene of which single copies are encoded on SpE and SpF. The 3’ ends of tRNA genes are typical targets for the integration of phages (att sites) (Campbell, 1996) and, correspondingly, we have designated the 45 terminal nucleotides of the tRNA gene plus an additional T as the attB site. The perfect match of chromosomal attB and plasmid attP sequences is shown in Fig. 6 (a). The attB site in the BmQ fragment is followed by 295 bp non-coding sequence and a 1356 bp large ORF that encodes an integrase (\textit{int}; GenBank accession no. AF285416) showing highest homology to the integrase of \textit{Actinobacillus actinomycetemcomitans} (AF006830). An att–spacer–\textit{int} contig is typical for phage attachment sites (Campbell, 1996), but unusual for a plasmid sequence.

Reversible chromosomal integration of plasmid at tRNA\textsuperscript{Lys} loci

Combinatorial PCR was applied to test the hypothesis that the 3’ sequence of the tRNA\textsuperscript{Lys} gene had been utilized by the clone K and C strains to incorporate their respective plasmids into the chromosome. Several primers were designed downstream and upstream of the

Some 5 products were obtained and sequenced. In all cases, the DNA from strains C, K, K1 and K2 by PCR. Single PCR chromosome–sequence contig in strains C, K1 and K2 reads hemA clone-specific plasmid sequence. In the junctions between species-specific chromosomal and other chromosomal tRNA genes in the PAO Lys (Fig. 6b). In other words, the strong sequence similarity between PAO and clone K strains ends about 800 bp downstream of the 3’ end of the tRNAlys gene and starts again at the att element (Fig. 4).

Our conclusion that the two tRNAlys genes are hotspots for the integration and excision of DNA was supported by the subculturing of single colonies of clone K isolates in vitro. Numerous descendants of a K1 ancestor carried pKLK106 DNA in the phnAB instead of the pilA region (for example strain K1*, Fig. 1), implying that the plasmid sequence was rapidly mobilized from one att site to the other. In summary, the genome rearrangements observed in sequential clone K isolates were based on the reversible integration of pKLK106 in either one or the other chromosomal tRNAlys gene. One site has also been employed by strain PAO to incorporate a DNA block encoding pyocin, transposases and IS elements, the other site by clone C to target its plasmid pKL102 into the chromosome.

**DISCUSSION**

The genome rearrangements in sequential clone K isolates from the airways of a CF patient who is homozygous for the major CFTR mutation ΔF508 (Tümmler & Kiewitz, 1999) were established by an integrated approach of mapping, sequencing and bioinformatics. The diverse genomic organization originated from the reversible integration of a 106 kb plasmid pKLK106 in either of two identical chromosomal recognition sites (attB). The target sites were identified within the 3’ end of lysine tRNA structural genes which are located in the pbnAB and pilA regions. The attB site close to pilA was also employed by strain C to integrate a highly similar plasmid, pKL102.

An 8.9 kb island was integrated into the PAO chromosome at the same tRNA recognition site in the pbnAB–oprL region as pKL106, albeit the discernible att 3’ sequence was truncated (attca). The annotation of the 8.9 kb insertion provides evidence that the DNA block was acquired by horizontal gene transfer (Fig. 4). Two different IS elements are located close to the borders of the element and encode transposases. One large ORF encodes a bacterioidal S-type pyocin. Otherwise, no function could be ascribed to the ORFs of the insertion. Twelve out of 17 identified ORFs upstream of and within the DNA block are orphan genes with no significant homology to any entry in the public sequence databases (Fig. 4), supporting the notion that a hyper-variable region should be devoid of (essential) housekeeping genes.

The 8.9 kb insertion is stably maintained in strain PAO and hence behaves like a pathogenicity island (Hacker et al., 1997; Hou, 1999). tRNAs play a role in the horizontal transfer of virulence gene clusters between different pathogens. An example is the tRNA€el locus of *E. coli* that has served as the site of integration of two distinct pathogenicity islands that are responsible for converting benign strains into uro- and enteropathogens (Blanc-Potard & Groisman, 1997). tRNAs play versatile roles in prokaryotes and eukaryotes. They are central components of the trans-

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**Fig. 5.** Comparison of the plasmids harboured by clone K and clone C. (a) Diagnostic BamHI fingerprints of the highly similar plasmids pKL106 of clone K and pKL102 of clone C. The conserved 2.4 kb BamHI fragment (BmQ) carrying the plasmid recombination site (attP) is indicated. (b) BamHI (outer circle) and SpeI (inner circle) maps of pKL102. SpeI fragment designations refer to Fig. 7a of Römling et al. (1997). BmQ is highlighted in black. Differential regions between pKL102 and pKL106 are shaded grey. Fragments SpAI’ and SpAQ of pKL102 are equivalent to the diagnostic 29 kb SpeI fragment in clone K isolates (see Fig. 2).
lational machinery and are essential for replication of retroviruses because the tRNAs bind to viral genomes through their 3′-end sequences and act as primers for initiation of viral replication (Hou, 1999). Many temperate bacteriophages integrate into the bacterial chromosome via site-specific integration at an attB site that is typically within, or overlaps with, the 3′ end of a tRNA gene (Campbell, 1996). In P. aeruginosa, the cytotoxin-converting temperate phage φCTX was found to integrate at a chromosomal serine tRNA gene (Hayashi et al., 1993; Nakayama et al., 1999).

Whereas the integration of phage genomes into their host chromosome at the 3′ end of tRNA genes has been demonstrated for numerous taxospecies (Campbell, 1996), reports that plasmids can recombine with host chromosome at the 3′ end of tRNA genes are rare. Examples are the elements pSAM2 from Streptomyces ambofaciens (Raynal et al., 1998) and pSE101 from Saccharopolyspora erythraea (Brown et al., 1994), which integrate into the chromosome at the 3′ end of the tRNAPro (pSAM2) and tRNAThr (pSE101) genes. Interestingly, pSAM2, pSE101 and our P. aeruginosa plasmids pKLC102 and pKLK106 all encode an integrase gene (int) in close vicinity to the attP end.

We compared the att sequences, their localization within the tRNA genes and the organization of the att–int sequencecontig of pKLK106 with that of conjugative mobile genetic elements pSAM2 (Raynal et al., 1998), pSE101 (Brown et al., 1994) and that of the temperate phages mv4 (Auvray et al., 1997; 1999), Sfi21 (Bruttin et al., 1997) and VWB (Van Mellaert et al., 1998) and the classical paradigm phage λ (Campbell, 1996). Amongst this set of selected examples, pKLK106 and pKLC102 are conspicuous by containing the largest spacer sequence of 295 bp between att and int. The att–int sequencecontigs of the plasmid-like mobile genetic elements are no more closely related to each other than they are to those of the phages. No general rules were seen with respect to the overlap of tRNA and att gene sequences, primary sequences and length of the att site, GC-contents and length of the spacer, and the coding sequence of int and its orientation with respect to att. All investigated examples share the localization of att at a tRNA gene locus and an adjacent int gene, but otherwise each analysed phage or conjugative genetic element is characterized by individual features of its primary sequence at the att locus.

In conclusion, by the analysis of sequential clone K isolates from CF airways, we have detected the reversible integration of a 106 kb plasmid at two identical att sites within the P. aeruginosa chromosome. The utilization of typical phage attachment sites by conjugative genetic elements could be one of the major mechanisms that allows P. aeruginosa to generate the mosaic genome structure of blocks of species-, clone- and strain-specific DNA. Our example demonstrates the potential impact of systematic genome analysis of sequential isolates from the same habitat on our understanding of the

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**Fig. 6.** att sequences of *P. aeruginosa*. (a) Sequence alignment of the conserved bacterial and plasmid att sites and flanking regions: the tRNA^{35}\textsuperscript{19} gene on SpE of PAO (pilaA region, see Fig. 3), the tRNA^{35}\textsuperscript{19} gene on the 169 kb SpeI fragment in strains K1 and K2 (oprt–phnAB region, see Figs 3 and 4), and the plasmid recombinase site (attR) of pKLC102 located on the 2.4 kb BamHI fragment (identical in pKLK106). The tRNA^{35}\textsuperscript{19} gene and conserved nucleotides within this gene are highlighted in black. A tRNA^{35}\textsuperscript{19} gene sequence from *E. coli* (GenBank accession no. K00282) is included for comparison; the anticodon is marked by asterisks. The 3′ terminal 45 bp of the tRNA^{35}\textsuperscript{19} gene with an additional T make up the attP and attR sites, respectively, indicated by the line. (b, c) Sequence alignments of the left junction (b) containing the complete tRNA^{35}\textsuperscript{19} gene, and the right junction (c) with the 46 bp att site underlined. The sequence alignments include both integration sites of the plasmids pKLK106 or pKLC102, either in the hemA–pilA region (strains K1, K2, C) or in the oprL–phnAB region of PAO (strain K) (see Figs 3 and 4). (c) In PAO the conserved 46 bp att site (underlined) in the oprL–phnAB region is truncated, leading to a 22 bp att* element (double underlined). The downstream sequence is conserved except for some nucleotide substitutions; one of them is an uncommon A to C transversion leading to the loss of a BglII site in strains K and K1/K2.
evolution of microbial genomes. The comparative genomics of strains that are collected from natural habitats over an extended period of time under well-documented sampling conditions provides robust primary data about genomic evolution in natural bacterial populations that are not biased by any heuristic assumptions or models and hence should withstand the test of time.

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