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

Claudia Kiewitz, Karen Larbig, Jens Klockgether, Christian Weinel and Burkhard Tümler

Author for correspondence: Burkhard Tümler. Tel: +49 511 5322920. Fax: +49 511 5326723. e-mail: tuemmler.burkhard@mh-hannover.de

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

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 & Tümler, 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

Abbreviation: CF, cystic fibrosis.
The GenBank accession numbers for the sequences reported in this paper are AF285416–AF285426.
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 repetitively from an attB site located within two separate copies of a lysine tRNA gene. Both tRNA loci are located adjacent to pilA (Römling 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^ cells ml^-1^), grown to late exponential phase were encapsulated into agarose plugs, lysed extensively with detergents and proteinase K, and the intact chromosomes were carried out, the fragments separated by PFGE and hybridized with cloned genes (Schmidt et al., 1996), and the reference strain PAO (DSM 1707) were included.

**Restriction mapping.** *P. aeruginosa* bacteria (5 × 10^9^ 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-DRIII 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, 1.5% 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-DRIII 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).

*SpeI* maps of strains K, K1 and K2 covering the *ttxA–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-DRIII 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 pLAFR3-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 where selected 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 *phnA* 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-

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

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5′–3′)</th>
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<tr>
<td><strong>phnA–oprL</strong></td>
<td></td>
</tr>
<tr>
<td><em>phn1f</em></td>
<td>AGGAGAAGTCCATGAGCATCCAGC</td>
</tr>
<tr>
<td><em>phn2r</em></td>
<td>TCGGGGACGAGTCTATCTTTCAGC</td>
</tr>
<tr>
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<td>CAAGGCCTAGAATGACTGTCCTTCC</td>
</tr>
<tr>
<td><em>phn4r</em></td>
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</tr>
<tr>
<td><em>phn5f</em></td>
<td>CGTTTGTCTGCGAAAGCTGCCAC</td>
</tr>
<tr>
<td><em>phn6r</em></td>
<td>TCCGCAAAACGGAAACACCATTC</td>
</tr>
<tr>
<td><em>phn7r</em></td>
<td>GAGAAGAATTGGCCTGGACCTTCG</td>
</tr>
<tr>
<td><em>phn8r</em></td>
<td>GAATTGTGCTCTATGGGAGGAGC</td>
</tr>
<tr>
<td><em>Sp1F1</em></td>
<td>ATCCGCTTCGGACTGAAACACCG</td>
</tr>
<tr>
<td><em>Sp2F1</em></td>
<td>GGGGTGGATTACCTCTGTTATCCGG</td>
</tr>
<tr>
<td><em>Sp3F1</em></td>
<td>GACAGACGTGCTGAAACACAGGAC</td>
</tr>
<tr>
<td><em>Sp4F1</em></td>
<td>AATTCAAGGGCGCAATCCGGAATA</td>
</tr>
<tr>
<td><strong>SpE primes</strong></td>
<td></td>
</tr>
<tr>
<td><em>SpEl1</em></td>
<td>CCAACTCTCAATCGGGGTATAGGCG</td>
</tr>
<tr>
<td><em>SpEl2</em></td>
<td>AGACGCCTGATGATCCGATCGGCG</td>
</tr>
<tr>
<td><strong>BmQ primes</strong></td>
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<tr>
<td><em>BmQ1f</em></td>
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</tr>
<tr>
<td><em>BmQ3r</em></td>
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<tr>
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</tr>
<tr>
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<td>CGTTTGTTGCGAAAGCTGCCAC</td>
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<tr>
<td><em>phn3f</em></td>
<td>CAAGGCCTAGAATGACTGTCCTTCC</td>
</tr>
<tr>
<td><em>phn2r</em></td>
<td>TCGGGGAGCAGGTCATATCTTCAGC</td>
</tr>
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grams BLAST N, BLAST X and BLAST P. Putative ORFs were identified with GeneMark (Besemer & Borodovsky, 1999). Pairwise sequence comparisons and multiple alignments were generated using CLUSTAL W (Thompson et al., 1994). Long-range restriction maps were constructed with the in-house program MasterMap. tRNA genes were identified by the program tRNA-scan-SE (Lowe & Eddy, 1997).

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 63% 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.
hybridize to, a 29 kb chromosomal \textit{Spe}I fragment in the clone K isolates. This 29 kb \textit{Spe}I fragment was mapped close to the breakpoint in strain K, but close to \textit{pilA}, 600 kb away in strains K1 and K2 (Fig. 3). This observation was the first hint that plasmid sequence could be integrated into the clone K chromosome at two distant sites. After isolation of pKLK106, its \textit{Bam}HI restriction fragment pattern turned out to be almost identical to that of the 102 kb clone C plasmid pKLC102 (Fig. 5), which occurs in clone C strains as an episomal plasmid and/or integrated into the clone C chromosome close to the \textit{pilA} locus (Römling et al., 1997) (Fig. 3). Hence we concluded that the related clone C and K plasmids could integrate into the same hypervariable region.

The recombination site of pKLC102 was mapped with cosmid-derived probes to fragment BmQ (Fig. 5b). To identify the plasmid–chromosome junction and putative targeting signals, the BmQ fragment was sequenced and searched for homologies in the completed PAO genomic sequence. Perfect matches of BmQ with PAO sequence were found in the hypervariable \textit{pilA} (\textit{Spe}I) and \textit{phnAB–oprL} regions (\textit{SpU}/\textit{SpF}). A 45 bp sequence of BmQ was identical to the 3’ region of a tRNA\textsubscript{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 (\textit{att} sites) (Campbell, 1996) and, correspondingly, we have designated the 45 terminal nucleotides of the tRNA gene plus 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 \textit{Bam}HI fragment (BmQ) forming the plasmid/chromosome junction in strain K. Triangles indicate oligonucleotide primers and asterisks depict matched pairs of complementary oligonucleotide primers.

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

Combinatorial PCR was applied to test the hypothesis that the 3’ sequence of the tRNA\textsubscript{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
attP site in BmQ and the two tRNA\textsubscript{Lys} genes in the PAO genome. One plasmid-derived and one chromosome-derived primer were combined to amplify chromosomal DNA from strains C, K, K1 and K2 by PCR. Single PCR products were obtained and sequenced. In all cases, the 3′ segment of the tRNA\textsubscript{Lys} gene was identified at the junctions between species-specific chromosomal and conserved plasmids pKLK106 of clone K and pKLC102 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 pKLC102. SpeI fragment designations refer to Fig. 7a of Römling et al. (1997). BmQ is highlighted in black. Differential regions between pKLC102 and pKLK106 are shaded grey. Fragments SpAI and SpAQ of pKLC102 are equivalent to the diagnostic 29 kb SpeI fragment in clone K isolates (see Fig. 2).

### 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 (Tümmeler & 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 PAO to incorporate a DNA block encoding pyocin, transposases and IS elements, the other site by clone C to target its plasmid pKLC102 into the chromosome.

An 8.9 kb island was integrated into the PAO chromosome at the same tRNA recognition site in the pbnAB–oprL region as pKLK106, albeit the discernible att 3′ sequence was truncated (att\textsuperscript{i}). 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 bactericidal 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\textsuperscript{Sel} locus of \textit{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-
Genome rearrangements in *P. aeruginosa*

(a) Sequence of bacterial and plasmid att sites

(b) Left junction of insertion (attL site)

(c) Right junction of insertion (attR site)

**Fig. 6.** att sequences of *P. aeruginosa*. (a) Sequence alignment of the conserved bacterial and plasmid att sites and flanking regions: the tRNA^{35′} gene on SpE of PAO pilA region, see Fig. 3, the tRNA^{35′} gene on the 169 kb SpeI fragment in strains K1 and K2 (optl–phnAB region, see Figs 3 and 4), and the plasmid recombination site (attP) of pKLC102 located on the 2.4 kb BamHI fragment (see Fig. 5). The tRNA^{35′} gene (identical in conserved nucleotides within this gene are highlighted in black. A tRNA^{35′} 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′} gene with an additional T make up the attP and attB sites, respectively, indicated by the line. (b, c) Sequence alignments of the left junction (b) containing the complete tRNA^{35′} 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.

Relational 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 chromosomal 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′ ends of the tRNA^{Pro} (pSAM2) and tRNA^{Thr} (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-spacer–int sequence contig 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 (Avray 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 sequence contigs 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
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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