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Pseudomonas aeruginosa genomic structure and diversity

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The *Pseudomonas aeruginosa* genome (G + C content 65–67%, size 5.5–7 Mbp) is made up of a single circular chromosome and a variable number of plasmids. Sequencing of complete genomes or blocks of the accessory genome has revealed that the genome encodes a large repertoire of transporters, transcriptional regulators, and two-component regulatory systems which reflects its metabolic diversity to utilize a broad range of nutrients. The conserved core component of the genome is largely collinear among *P. aeruginosa* strains and exhibits an interclonal sequence diversity of 0.5–0.7%. Only a few loci of the core genome are subject to diversifying selection. Genome diversity is mainly caused by accessory DNA elements located in 79 regions of genome plasticity that are scattered around the genome and show an anomalous usage of mono- to tetradecanucleotides. Genomic islands of the pKLC102/PAGI-2 family that integrate into $tRNA^{Lys}$ or $tRNA^{Gly}$ genes represent hotspots of inter- and intraclonal genomic diversity. The individual islands differ in their repertoire of metabolic genes that make a large contribution to the pangenome. In order to unravel intraclonal diversity of *P. aeruginosa*, the genomes of two members of the PA14 clonal complex from diverse habitats and geographic origin were compared. The genome sequences differed by less than 0.01% from each other. One hundred ninety-eight of the 231 single nucleotide substitutions (SNPs) were non-randomly distributed in the genome. Non-synonymous SNPs were mainly found in an integrated Pf1-like phage and in genes involved in transcriptional regulation, membrane and extracellular constituents, transport, and secretion. In summary, *P. aeruginosa* is endowed with a highly conserved core genome of low sequence diversity and a highly variable accessory genome that communicates with other pseudomonads and genera via horizontal gene transfer.

Keywords: *Pseudomonas aeruginosa*, genome, genomic island, core genome, accessory genome, clonal complex, oligonucleotide signature

INTRODUCTION

The genetic repertoire of *Pseudomonas aeruginosa* reflects the lifestyle of this ubiquitous bacterial species. *P. aeruginosa* strains are found in various environmental habitats as well as in animal and human hosts, where they can act as opportunistic pathogens. The colonization of this broad spectrum of habitats goes along with the ability to exploit many different nutrition sources and a high potential for adaptation to new (or changing) environmental conditions (Ramos, 2004).

The metabolic versatility is provided by genes encoding not only the enzymes participating in metabolic pathways, but also by a very high number of transcriptional regulators and two-component regulatory systems. More than 500 regulatory genes were identified in the genome of strain PAO1 (Stover et al., 2000). The genomes of *P. aeruginosa* strains are larger than those of most sequenced bacteria. Within the species, the genome size varies between 5.5 and 7 Mbp (Schmidt et al., 1996; Lee et al., 2006).

The divergence in genome size is caused by the so-called accessory genome. The major part of the genome, the core genome, is found in all *P. aeruginosa* strains with the respective DNA generally collinearly arranged (Römling et al., 1995). The core genome, with few exceptions of loci subject to diversifying selection, is highly conserved among clonal complexes and shows sequence diversities of 0.5–0.7% (Spencer et al., 2003; Lee et al., 2006; Cramer et al., 2011). The accessory genome consists of extrachromosomal elements like plasmids and of blocks of DNA inserted into the chromosome at various loci. The elements of the accessory genome can be present in subgroups of the *P. aeruginosa* population but may also occur only in single strains (Klockgether et al., 2007; Wiehlmann et al., 2007). The individual composition of the accessory genome accounts for most intra- and interclonal genome diversity in *P. aeruginosa*. The elements of the accessory genome were apparently acquired by horizontal gene transfer from different sources including other species or genera. Upon integration into the host chromosome they appear as "foreign" blocks in the core genome. Therefore, a *P. aeruginosa* chromosome is often described as a mosaic structure of conserved core genome frequently interrupted by the inserted parts of the accessory genome.

The individual mosaics also show remarkable plasticity. Ongoing acquisition of new foreign DNA as well as larger or smaller deletion events, mutations of single nucleotides and even chromosomal inversions (Römling et al., 1997; Ernst et al., 2003; Kresse et al., 2003; Smith et al., 2006; Klockgether et al., 2010; Cramer et al., 2011) – all of them potentially affecting parts of the core and/or the accessory genome – continuously modify the genome,

modulate the *P. aeruginosa* strain's phenotype and differentiate it from others.

Genome diversity of *P. aeruginosa* was initially analyzed by low-resolution physical mapping techniques (Schmidt et al., 1996; Römling et al., 1997). Thanks to progress in DNA sequencing technologies *P. aeruginosa* genomes can nowadays be compared by the base (Kung et al., 2010; Silby et al., 2011).

GENOME SEQUENCES

Pseudomonas aeruginosa is ubiquitous in aquatic habitats and colonizes animate surfaces of humans, animals and plants. Complete genome sequences, however, are so far only available for *P. aeruginosa* isolates from human infections (**Table 1**).

The first complete genome sequencing was performed for strain PAO1 (Stover et al., 2000), derived from an Australian wound isolate from the 1950s. The PAO1 strain has been and is still the major reference for genetic and functional studies on *P. aeruginosa*. The PAO1 genome consists of a 6.264-Mbp circular chromosome encoding 5,570 predicted protein coding sequences. Sequence and annotation are deposited at the National Center for Biotechnology Information (NCBI) genome database (Refseq. no. NC_002516) and in the *Pseudomonas* Genome Database (Winsor et al., 2009), which also documents ongoing annotation updates. Thanks to the recently developed deep cDNA sequencing more and more non-coding RNAs are currently being identified in bacterial genomes, and thus we can expect a large number of non-coding genes to be added to the annotation of *P. aeruginosa* genomes as has been executed for *Helicobacter pylori* and *Pseudomonas putida* (Sharma et al., 2010; Frank et al., 2011).

The second *P. aeruginosa* genome sequence was published for the ExoU-positive strain PA14 (NC_008463, Lee et al., 2006), a clinical isolate displaying higher virulence than PAO1. Fifty-four PAO1 regions of at least one open reading frames (ORFs) are absent in the PA14 genome, and 58 PA14 regions are absent in PAO1 including the PA14 pathogenicity islands PAPI-1 and PAPI-2 (He et al., 2004).

LESB58, a so-called "Liverpool epidemic strain," was found to be highly transmissible among CF-patients and displayed the potential to cause severe infections even in non-CF human hosts (Cheng et al., 1996; McCallum et al., 2002). The LESB58 genome (NC_011770) contains previously unknown accessory genome elements (Winstanley et al., 2009).

PA7 is a clinical isolate from Argentina with a notably unusual antimicrobial resistance pattern. Strain PA7 (NC_009656) shares only 93.5% nucleotide identity in the core genome with the other sequenced strains confirming the previous assignment of strain PA7 as a taxonomic outlier within the species *P. aeruginosa* (Roy et al., 2010).

Almost complete genome sequences are also available for strains 2192 (NZ_AAKW00000000), C3719 (NZ_AAKV00000000), PACS2 (NZ_AAQW00000000; Mathee et al., 2008), and 39016 (AEEX00000000; Stewart et al., 2011). Eight additional *P. aeruginosa* genome sequences are listed at NCBI as "In Progress" (last checked on February 23rd, 2011) and numerous *P. aeruginosa* projects are deposited in the European Nucleotide Archive (ENA) hosted by EMBL-EBI[1]. With decreasing costs and increasing speed of sequencing we can expect an avalanche of novel *P. aeruginosa* genome sequence data. Published examples are the comparative sequencing of PAO1 sublines of divergent metabolic and virulence phenotypes (Klockgether et al., 2010), the identification of *de novo* mutations conferring antimicrobial resistance (Moya et al., 2009), the analysis of genomic gradients of sequence diversity in a pool of clinical isolates (Dötsch et al., 2010), and the intraclonal microevolution in the cystic fibrosis lung (Cramer et al., 2011).

THE ACCESSORY GENOME

The accessory genome consists of DNA elements from within the range of a few hundred bases to more than 200 kbp. The minimum size of an accessory element was defined as a block of at least four contiguous ORFs that are not conserved in all *P. aeruginosa* (Mathee et al., 2008). Thirty-eight to 53 accessory elements were identified in the completely sequenced *P. aeruginosa* genomes (**Table 2**). The PAO1 genome only contains inserts of 14 kbp or smaller (Mathee et al., 2008), whereas the LESB58 genome harbors five genomic islands and five inserted prophages of 14–111 kbp in size (Winstanley et al., 2009). **Table 3** lists the subset of genomic islands that were analyzed in detail *in silico* and/or in wet lab experiments.

Within the chromosomally integrated islands, very often phages, transposons, or IS-elements are found indicating that the majority of the accessory genome originates from mobile DNA elements which have been acquired and kept by the host strain. Many elements were irreversibly fixed by secondary mutation or deletions, but a few others have retained their mobility and can still leave the chromosomal insertion site and be transferred elsewhere, as shown for the elements PAPI-1 (Qiu et al., 2006) and pKLC102 (Klockgether et al., 2007). For a detailed description of the different types of accessory elements [integrative and conjugative elements (ICEs), prophages, transposons, etc.], the reader is referred to the recently published review by Kung et al. (2010).

The acquisition of the elements of the accessory genome from other taxa is not only evident from the gene contents with its overrepresentation of mobile DNA elements, but also from global

[1]http://www.ebi.ac.uk/ena/

Table 1 | Features of sequenced *P. aeruginosa* strains.

Strain	PAO1	PA14	PA7	LESB58	PACS2	2192	C3719	39016
Source	Wound	Clinical	Clinical	CF-patient	Clinical	CF-patient	CF-patient	Keratitis
Genome size (Mbp)	6.264	6.538	6.588	6.602	6.492	6.905	6.222	6.667
GC-content (%)	66.6	66.3	66.5	66.5	66	66.2	66.5	66
No. of protein coding ORFs	5570	5892	6286	5925	5676	6191	5578	6401

Table 2 | Regions of genome plasticity (RGP) in seven sequenced *P. aeruginosa* genomes.

RGP		Flanking loci		Strain						
	Insertion Site	In PAO1[1]	In PA14[1]	PAO1	PA14	LESB58	PA7	2192	C3719	PACS2
RGP1		0201/0208	02530/02550	*			i		*	*
RGP2	$tRNA^{Arg}$	0256/0264	03160/03420	*	i	*	i	*	*	
RGP3		0611/0629	07960/08160	*	*	i[2]	i		+	+
RGP4		0641/0648	08300/08330	*	i	i[2]	i	*	i	i
RGP5	$tRNA^{Gly}$	0714/0730	55100/54830	i	i		i	i		i
RGP6	tmRNA	0819/0827	53680/53560	i	i	i	i	i	i	i
RGP7	$tRNA^{Lys}$	0976/0988	51670/51510	i	i		i			
RGP8	$tRNA^{Ser}$	1013/1014	51240/51220				i		i	
RGP9[3]		1087/1092	50340/50290	*	*	*	i	i	*	i
RGP10		1191/1192	49040/48870		i	i	i			
RGP11		1222/1225	48520/48440	*	i	*	i	*	*	*
RGP12		1243/1244	48160/48150				i	i		
RGP13		1367/1373	46630/46490	i	i	i	i	i		
RGP14		1375/1376	46470/46540		i				i	
RGP15		1377/1394	46440/46390	i	i		i[4]	i	i	i
RGP16		1530/1531	44650/44640		i[5]				i	i
RGP17	$tRNA^{His}$	1796/1797	41350/41280				i	i		
RGP19		1964/1965	39130/39110					i		
RGP20		2024/2070	38340/37730	i	*	*	i	i		i
RGP21		2099/2107	37360/37350	*				*		
RGP22		2181/2187	36370/36360	*		*	i		*	*
RGP23		2217/2235	36050/35690	i	i	i	i	i	i	i
RGP24		2422/2423	33370/33290		i		i	i	*	*
RGP25		2455/2464	32860/32770	i	i	i	i	i	i	i
RGP26	$tRNA^{Leu}$	2570/2571	31290/30840		i		i			
RGP27	$tRNA^{Gly}$	2583/2584	30700/30670		i	i	i			i
RGP28	$tRNA^{Pro}$	2727/2737	28895/28730	i	i	i	i	i	i	i
RGP29	$tRNA^{Gly}$	2817/2820	27710/27590	+	*	*	+	i	*	*
RGP30		2950/2951	25900/25880					i		
RGP31[6]		3141/3160	23470/23360	i	i	i	i	*	i	*
RGP32		3222/3223	22560/22490		i					
RGP33		3239/3240	22290/22075		i					
RGP34		3496/3515	18870/18860	i						
RGP35		3536/3537	18620/18610					i		
RGP36		3768/3769	15670/15340		i	i				i
RGP37		3865/3870	13990/13850	*	i			*		
RGP38		4162/4163	10130/10040		*		*			
RGP39		4190/4196	09700/09690	*		*		*	*	*
RGP41	$tRNA^{Lys}$	4541/4542	58900/60190		i			i	i	i
RGP42	$tRNA^{Met}$	4673/4674	61820/61840	i[7]			i	i		i
RGP43		2770/2773	28280/28220	*[8]	i	*[8]	i	*[8]	*[8]	*[8]
RGP44		4100/4108	10850/10820	*	i	*	*	*	*	*
RGP46		0041/0042	00510/00530		i	i	i	i	i	
RGP47		1149/1153	49530/49500	i	i	*		*	i	*
RGP48		1238/1242	48240/48170	*	*			*		*
RGP50		1655/1656	43110/43050		i					
RGP52		1934/1940	39500/39460	i	i	i	i	i	i	i
RGP53		2332/2337	34450/34440	*		*		*	*	*
RGP56		2793/2795	28000/27980	*	*	+	i	+	*	+[9]
RGP58	$tRNA^{Arg}$	3366/3368	20560/20490	*	i	i	*	*	*	i
RGP60[10]	$tRNA^{Thr}$	4524/4526	58700/58750	i	*	+	i	+	*	i

(Continued)

Table 2 | Continued

RGP		Flanking loci		Strain						
	Insertion Site	In PAO1[1]	In PA14[1]	PAO1	PA14	LESB58	PA7	2192	C3719	PACS2
RGP62	$tRNA^{Phe}$	5149/5150	68000/68040							i
RGP63		0069[11]	00810[11]				i			
RGP64		0278[11]	03620[11]				i			
RGP65		0377/0378	04940/04950				i			
RGP66	$tRNA^{Met}$	0574/0575	07450/07500		i		i			
RGP67		3858/3859	14080/14100				i			
RGP68[12]		3840/3844	14290/14340	*	i	*	i	*	*	*
RGP69		3714/3715	16340/16350				i			
RGP70	$tRNA^{Pro}$	3031/3032	24860/24880				i			
RGP71		2650/2651	29820/29830			*	i		*	*
RGP72	$tRNA^{Cys}$	2581/2582	30710/30730				i			
RGP73[13]		2397/2403	33600/33690	*	*	i	+	i	+	+
RGP74		2201/2202	36230/36250				i			
RGP75		1579/1580	44070/44080				i			
RGP76		1425/1428	45980/46010	*	*	*	i	*	*	*
RGP77		1397/1398	46330/46350				*			*
RGP78		4466/4467	57980/57990				i			
RGP79		5290/5291	69840/69850				i			
RGP80		5454/5460	72000/72060	*	*	*	i	*	*	*
RGP81		4138/4139	10420/10380			i				
RGP82		3663/3664	16980/16970			i				
RGP83		3463/3464	19330/19320	i[14]		i		i[14]	*[14]	*[14]
RGP84	$tRNA^{Ser}$	2603/2604	30430/30410			i	[15]			
RGP85		2593/2594	30550/30560			i	[15]			
RGP86		0831/0832	53510/53520			i				
RGP87	$tRNA^{Thr}$	5160/5161	68140/68170					i		i
RGP88		3961[16]	12630[16]							
RGP89		3834/3836	14440/14390	*	i	*	+	i	*	+

*Differentiation of accessory elements in the RGPs: i, strain-specific accessory element; *or +, identical accessory elements in two or more strains. RGPs 1–62 were defined by Mathee et al. (2008) and RGPs 63–80 by Roy et al. (2010). The novel RGPs 81–89 were extracted from the sequences of genomic islands in strain LESB58 (RGP 81–86; Winstanley et al., 2009) and strain PSE9 (RGP 87–89; Battle et al., 2009).*

[1] Insertions are designated by the numbers of the flanking loci in the PAO1 and PA14 genomes (e.g., 0201 is PA0201, 02530 is PA14_02530).

[2] Insertion LESPP-1 between PA0612 and PA0648 homologs comprises RGP3 and RGP4.

[3] Region containing flagellin glycosylation genes (replacement island).

[4] Partial duplication of sequence of the core genome (between RGP27 and RGP28).

[5] No annotated ORF in this insertion.

[6] Region contains O-antigen gene cluster (replacement island).

[7] No insertion in PAO1 reference sequence but in variants PAO1-DSM and MPAO1 (Klockgether et al., 2010).

[8] Identical sequence with discordant ORF annotation for the different strains.

[9] Identical sequence with discordant annotation for PACS2 versus LESB58 and 2192.

[10] Region contains pilA gene (replacement island).

[11] Homologous ORF in PA7 disrupted by the insertion.

[12] Insertion contains exoS gene in PAO1, LESB58, 2192, C3719, and PACS2.

[13] Region contains pyoverdine synthesis gene cluster (replacement island).

[14] < 1 kb insertion in PAO1, 2192, C3719, and PACS2 with no predicted ORF.

[15] Insertion in PA7 comprises RGP84 and RGP85.

[16] Homologous ORF in strain PSE9 disrupted by PAGI-7.

parameters like the oligonucleotide signature. The segments of the core genome share the same oligonucleotide usage, whereas the constituents of the accessory genome exhibit a divergent G + C content and oligonucleotide usage (Reva and Tümmler, 2004, 2005). In the genome atlas of *P. aeruginosa* LESB58 (**Figure 1**), the regions with an anomalous tetranucleotide composition and an underrepresentation of common octa- to tetradecanucleotides coincide with the segments of the accessory genome. **Figure 2**

Table 3 | Genomic islands in *P. aeruginosa* strains described in literature.

Genomic island	Host strain	Size (kb)	RGP locus	Reference
PAPI-1	PA14	108	41	He et al. (2004)
PAPI-2	PA14	10.8	7	He et al. (2004)
LES-prophage 1	LESB58	14.8	3 and 4	Winstanley et al. (2009)
LES-prophage 2	LESB58	42.1	81	Winstanley et al. (2009)
LES-prophage 3	LESB58	42.8	82	Winstanley et al. (2009)
LES-prophage 4	LESB58	26.8	83	Winstanley et al. (2009)
LES-prophage 5	LESB58	39.9	84	Winstanley et al. (2009)
LES-prophage 6	LESB58	7.6	10	Winstanley et al. (2009)
LESGI-1	LESB58	46.4	28	Winstanley et al. (2009)
LESGI-2	LESB58	31.7	85	Winstanley et al. (2009)
LESGI-3	LESB58	110.6	27	Winstanley et al. (2009)
LESGI-4	LESB58	39.4	23	Winstanley et al. (2009)
LESGI-5	LESB58	29.4	86	Winstanley et al. (2009)
pKLC102	C, SG17M	103.5	41	Klockgether et al. (2004)
PAGI-1	X24509	48.9	23	Liang et al. (2001)
PAGI-2	C	105	29	Larbig et al. (2002)
PAGI-3	SG17M	103.3	29	Larbig et al. (2002)
PAGI-4	C	23.4	7	Klockgether et al. (2004)
PAGI-5	PSE9	99.3	7	Battle et al. (2008)
PAGI-6	PSE9	44.4	87	Battle et al. (2009)
PAGI-7	PSE9	22.5	88	Battle et al. (2009)
PAGI-8	PSE9	16.2	62	Battle et al. (2009)
PAGI-9	PSE9	6.6	89	Battle et al. (2009)
PAGI-10	PSE9	2.2	25	Battle et al. (2009)
PAGI-11	PSE9	2	52	Battle et al. (2009)
ExoU-A	6077	81.2	7	Kulasekara et al. (2006)
ExoU-B	19660	29.8	7	Kulasekara et al. (2006)
ExoU-C	X13273	3.7	7	Kulasekara et al. (2006)

shows the genome distribution of the most abundant 8- to 14mers in *P. aeruginosa* LESB58 (Davenport et al., 2009). Regions that lack these strain- or taxon-specific words represent those parts of the accessory genome that is most foreign from the core.

REGIONS OF GENOME PLASTICITY

Elements of the accessory genome are located in all sections of the *P. aeruginosa* chromosome, not concentrated in some regions. Nevertheless, the uptake of accessory DNA apparently did not occur completely at random but at specific genomic loci that are prone to integration of special mobile elements.

A comprehensive comparison of the genomes of strains PAO1, PA14, 2192, C3719, and PACS2 (Mathee et al., 2008) led to the definition of so-called "regions of genome plasticity" (RGPs). Mathee and co-workers searched for segments of DNA not conserved in all five genomes and designated any region containing a block of four or more contiguous ORFs that is missing in at least one of the genomes as an RGP. For each of these RGPs they defined the DNA contained in the accessory blocks and the ORFs annotated within. Also the RGP flanking ORFs conserved in all five strains were listed, referred to as "anchors," which describe the genomic site used for the integration of the foreign DNA.

The approach by Mathee et al. (2008) appears reasonable to describe accessory and core genome of *P. aeruginosa* strains, although small insertions are ignored and deletions affecting the core genome in some, but not all, compared strains will misassign the respective segment to the accessory genome. A secondary check of the oligonucleotide usage will correct these false positives.

Mathee et al. (2008) initially defined 52 RGPs (no. 1–62 in **Table 2**). With the advent of the PA7 genome sequence, a further 18 elements were identified (RGPs 63–80; Roy et al., 2010).

Table 2 moreover lists the novel RGPs 81–89 that comprise yet unknown RGPs from strains LESB58 (Winstanley et al., 2009) and PSE9 (Battle et al., 2009).

On average each sequenced *P. aeruginosa* strain carries about 40 RGPs with insertions. The outlier was strain PA7 with 53 occupied RGPs. tRNA genes serve as integration sites for 20 RGPs. The 3′ end of tRNA genes and the subsequent nucleotides are known to serve as integration sites for ICEs and phage-like elements (Dobrindt et al., 2004). In the majority of RGPs, however, other target sequences had been utilized for the insertion corresponding with the diverse type and origin of the elements of the accessory genome of *P. aeruginosa* (Kung et al., 2010). Most target sequences are located in intergenic regions, but in three RGPs a single ORF was disrupted (RGPs 63, 64, and 88; **Table 2**). Interestingly, insertions in each of these three RGPs were only detected for a single strain so far, while in all other tested genomes the non-fragmented anchor-ORF was present.

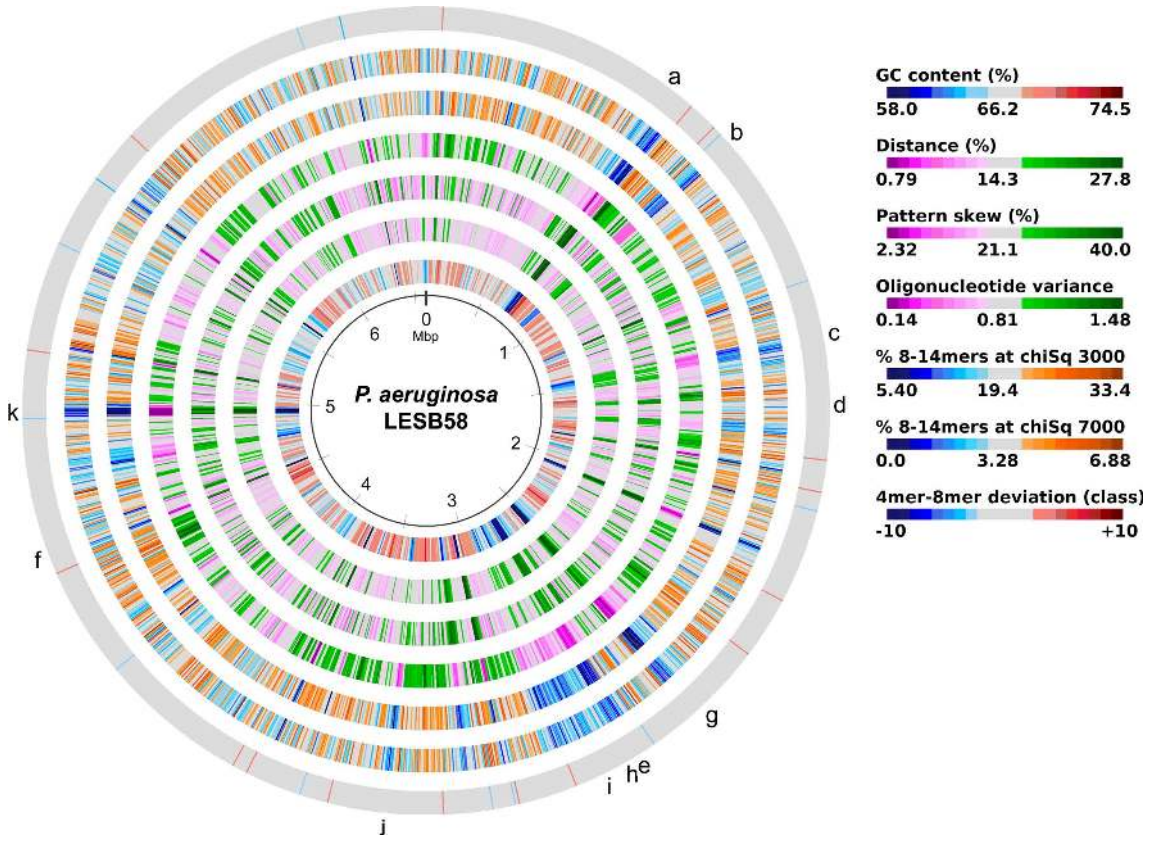

FIGURE 1 | Genome atlas representations of G + C content, tetranucleotide parameters and overrepresented 8- to 14mers in *P. aeruginosa* LESB58 (Davenport et al., 2009). Increasing divergence from average (up to an extreme value at ±3 SD) is indicated by progressively darker colors. G + C content and the three tetranucleotide parameters are plotted on the innermost four rings. Distance (second innermost circle) is the distance between global and local sliding window tetranucleotide patterns, pattern skew (third innermost circle) is the distance between tetranucleotide rankings on direct and reverse strands, and oligonucleotide variance (fourth innermost circle) is the numerical variance of oligomers, where a lower value indicates tetramer usage is more highly restricted (for example in repeat regions). Rings 5 (χ^2 threshold 3000) and 6 (χ^2 threshold 7000) display the number of bases occupied by overrepresented 8- to 14mers in a certain region, with overlaps only counted once, as a percentage. The outermost ring shows the difference (in classes) between a tetranucleotide parameter, oligonucleotide variance, and the 8- to 14mers in ring 5. Figures were created with JCircleGraph. Letters at the outermost ring indicate the regions of the six identified prophages (a–f) and five genomic islands (g–k; Winstanley et al., 2009).

Three regions show an unusual local genome structure. Strains LESB58 and PA7 each carry hybrids of two adjacent RGPs. Moreover, in strain LESB58 a 137-kbp segment of the core genome 3′ to RGB15 was transposed upstream by 83 genes (84.3 kbp; **Figure 3**). No repeats flanking the segment or mobility-related genes such as transposase- or integrase-coding genes were identified so that the underlying mechanism of the transposition remains elusive.

THE pKLC102/PAGI-2 ICE FAMILY

Among the genomic islands of the *P. aeruginosa* accessory genome, members of the pKLC102/PAGI-2 family are highly prevalent. They represent a special group of ICEs that can be described as semi-conserved elements, as they generally consist of individual DNA blocks and sets of genes common to all members (Klockgether et al., 2008; Kung et al., 2010). pKLC102/PAGI-2 family islands have been detected in various bacterial species and genera, mainly in β- and γ-proteobacteria. The fact that a set of genes is conserved among all family members indicates a common origin from an ancient ancestor (Mohd-Zain et al., 2004). This conserved gene set accounts for structural and mobility-related features and conjugal transfer. Individual genes within the islands can encode a broad spectrum of different functions, among them catabolic pathways as well as virulence effectors. Existence of free episomal forms and/or transfer to other strains, even across species barriers, have been monitored for several pKLC102/PAGI-2-like islands, thus confirming their role for (ongoing) evolution of bacterial genomes and, due to the different "cargo" provided by these elements to the host strains, for the genome diversification within bacterial species and emergence of subgroup- or strain-specific phenotypes. For a detailed summary of the role of the common "backbone" genes for integration, mobilization and transfer of pKLC102/PAGI-2-like elements, the reader is referred to the recent review by Kung et al. (2010).

The role of pKLC102/PAGI-2-like islands within the *P. aeruginosa* accessory genome, and thus their contribution to genome

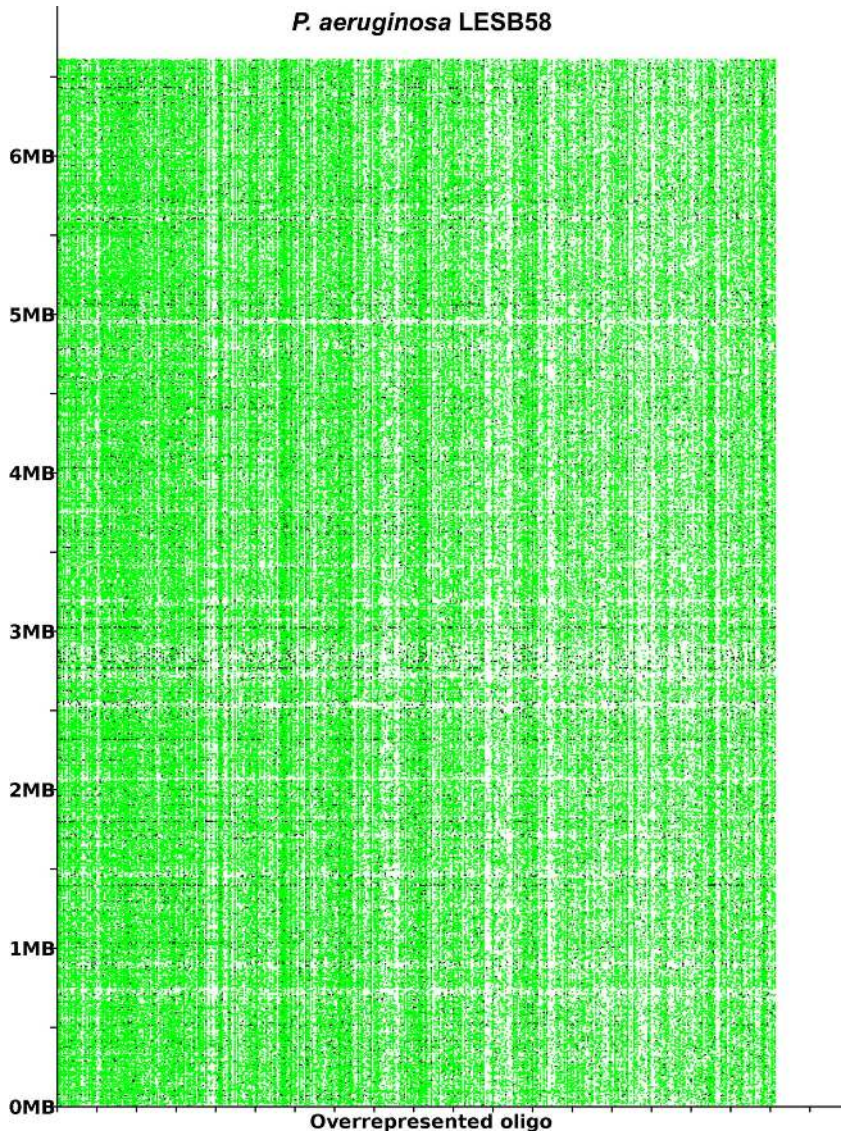

FIGURE 2 | The most overrepresented 8- 14 bp oligomers in *P. aeruginosa* LESB58 sorted by decreasing χ^2 values. The genome position of each oligo is plotted on the *y*-axis. A black dot is printed where an oligonucleotide occurs in non-coding regions and a green dot where an oligonucleotide occurs in coding regions. The figure was created with the program OligoViz (Davenport et al., 2009). The majority of the overrepresented 8–14 bp oligomers is located in coding sequences distributed all over the genome; only in the few cases of white vertical lines the respective oligonucleotide clusters in a few genome positions. Horizontal white lines indicate regions with an atypical oligonucleotide usage that lack these strain- or taxon-specific words and represent those parts of the accessory genome that are most foreign from the core.

diversity, is illustrated by the abundance of many different islands of this family within the population. Hybridization results have indicated the presence of such islands in a majority of strains isolated from different habitats (Klockgether et al., 2007; Wiehlmann et al., 2007). Similarly, searching the available *P. aeruginosa* genome sequences for the typically conserved genes revealed their presence in all strains but PAO1.

Six of the islands listed in **Table 3** are members of that family: pKLC102, PAPI-1, PAGI-5, PAGI-2, PAGI-3, and LESGI-3. All of them are between 99 and 110 kbp in size. Clusters of typically conserved backbone genes were also detected in smaller islands like PAGI-4 or ExoU-A. As significant parts of the backbone, however, were missing, it was hypothesized that PAGI-4 and ExoU-A represent remaining fragments of formerly complete PAGI-2/pKLC102-like islands that underwent recombination and deletion events resulting in the loss of smaller (ExoU-A) or bigger parts (PAGI-4) of the original elements (Klockgether et al., 2004; Kulasekara et al., 2006).

The mentioned *P. aeruginosa* islands split up into two subtypes: PAGI-2-like islands (PAGI-2, PAGI-3, and LESGI-3) contain a phage P4-related integrase gene and are inserted at $tRNA^{Gly}$ genes in RGPs 27 or 29. The well described *clc* element providing

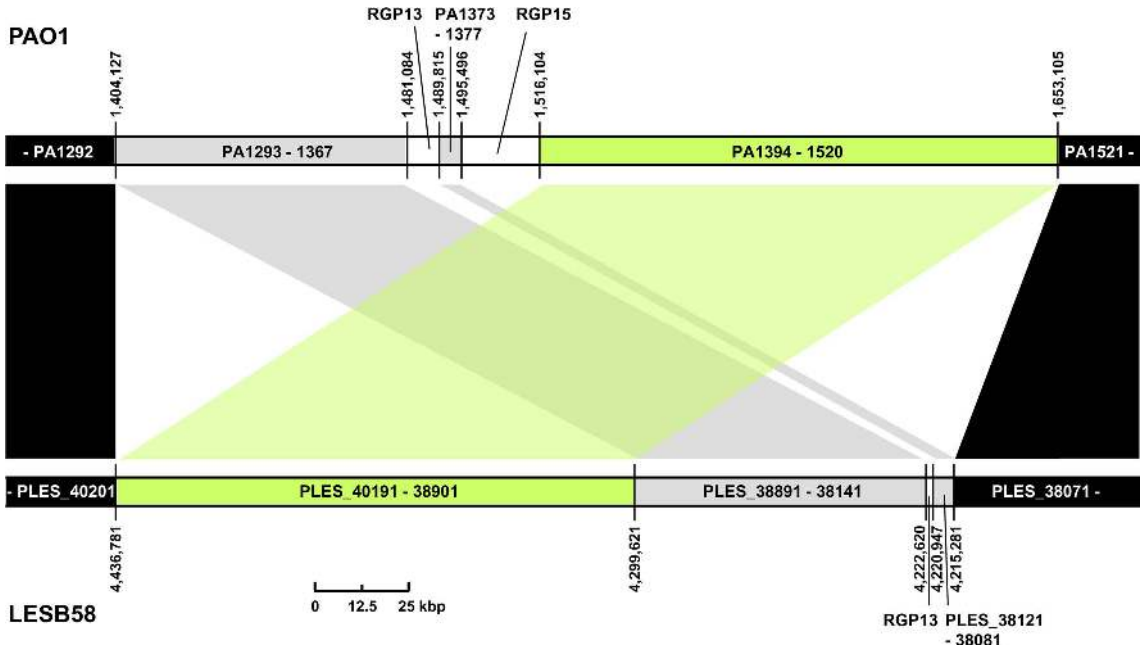

FIGURE 3 | Transposition of core genome DNA in LESB58. The genomic region with different core genome architecture is shown for strains PAO1 and LESB58. One hundred thirty-seven kbp of DNA (green) are located upstream of other core genome DNA blocks (gray) in LESB58 while occurring downstream of them in PAO1 (and other genomes). Surrounding core genome DNA arranged collinearly in both strains is shown in black, strain-specific insertions are represented by white areas. Genome coordinates of the borders of the core genome DNA blocks and numbers of the ORFs within are given for both strains. Accessory DNA blocks are described by the RGP number (see **Table 2**).

features for metabolizing chlorinated aromatic compounds could be assigned to that subtype as well. Present in other *Pseudomonas* species as well as in *Ralstonia* and *Burkholderia* strains, transfer of *clc* to *P. aeruginosa* PAO1 by conjugation was shown *in vitro* (Gaillard et al., 2008). Upon transfer, genomic integration occurred at the usual $tRNA^{Gly}$ genes in RGP27 or RGP29.

The pKLC102-subtype islands (pKLC102, PAPI-1, PAGI-5) are endowed with a XerC/XerD-like integrase gene, and the two copies of a $tRNA^{Lys}$ gene in RGP7 and RGP41 can be used as insertion sites. Transfer of pKLC102-like elements from one RGP to the other has been demonstrated (Kiewitz et al., 2000; Qiu et al., 2006). The "fragmentary" pKLC102-like islands PAGI-4 and ExoU-A are also located in RGP7. The $tRNA^{Lys}$ gene in RP7 is also the insertion site for islands carrying the virulence-associated *exoU* gene and its cognate chaperone *spcU* gene, ExoU-B, ExoU-C, and PAPI-2. Although DNA typical for pKLC102-like islands is scarce in these *exoU*-positive islands, the common insertion site and a few motifs within their sequence indicate a descent from a pKLC102-like element as hypothesized for ExoU-A (Kulasekara et al., 2006).

Kung et al. (2010) described the two subtypes as two families of *P. aeruginosa* ICEs. Due to the conserved function and synteny of the backbone genes, however, we prefer to consider them as members of one family with common ancestry (Klockgether et al., 2007, 2008). The pKLC102/PAGI-2-like islands share 35 conserved orthologs with a variable degree of amino acid identity between 35 and 100%.

Divergent evolution from the ancestor might have caused the early formation of the two pKLC102- and PAGI-2 subtypes that exhibit higher average identity values among the conserved backbone genes and each carry a subfamily-specific set of genes (**Figure 4**). Eleven genes were specific for the PAGI-2-subtype and 39 genes specific for the pKLC102- subtype including a cluster of conjugative type IV sex pilin genes (Klockgether et al., 2004; Carter et al., 2010). Thus, pKLC102-/PAGI-2-family islands appear as mosaic pieces in *P. aeruginosa* genomes while they are small mosaics themselves, composed of conserved backbone, subtype-specific, and individual cargo genes.

Due to their size, islands of this family can represent a major portion of the accessory genome. Strains with one or two large pKLC102/PAGI-2-family elements are common, but higher numbers per genome are possible. *P. aeruginosa* strain C harbors PAGI-2 and pKLC102, but two more sets of backbone ORFs have been identified in the chromosome indicating four related elements in total, with an overall DNA sequence length of more than 360 kbp (own unpublished data). Of the seven genomes presented in **Table 2**, six contain large pKLC102/PAGI-2-family islands. Strains PA14, C3719, and PA7 each harbor one pKLC102-like island in RGP41 or, in case of PA7, in RGP7. LESB58 also contains one island, but of the PAGI-2 subtype (LESGI-3 in RGP27). Two islands each are located in the 2192- and the PACS2 genomes. Both strains also harbor a pKLC102-like insertion in RGP41 and a PAGI-2-related island, which is in RGP29 for strain 2192 and in RGP27 in PACS2.

The island in 2192 inserted at RGP29 is a nearly identical copy of PAGI-2 itself but is interestingly accompanied by another island of comparable size, the so-called Dit-island which is distinct from the

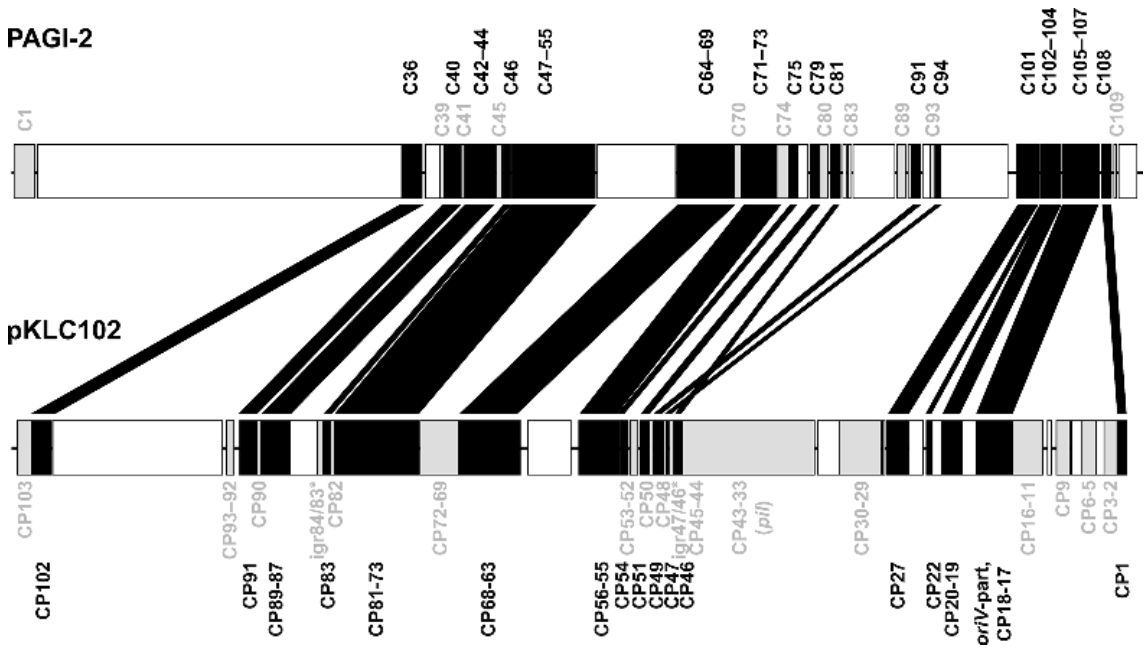

FIGURE 4 | Conserved genes in pKLC102-/PAGI-2-like genomic islands. PAGI-2 (Larbig et al., 2002) and pKLC102 (Klockgether et al., 2004) were chosen as representatives for the respective subtypes among the pKLC102-/PAGI-2 family. The annotated ORFs are labeled according to their conservation. ORFs appearing in all *P. aeruginosa* islands of this family ("backbone genes") are shown in black. ORFs conserved within one of the subtypes are colored in gray. White blocks represent ORFs specific for the single islands ("individual cargo"). Intergenic regions (igr) marked with an asterisk indicate loci with no ORF annotated for pKLC102 but for the highly homologous sequences in other islands from this subtype. Please note that ORF C105 of PAGI-2 is homologous to DNA in pKLC102 described as a part of the replication origin *oriV* of this element. The other part of *oriV* containing 16 57 bp repeats (Klockgether et al., 2004) is not conserved among the island family, not even in other islands from the pKLC102 subtype.

pKLC102/PAGI-2 family (Mathee et al., 2008). Thus an extremely large insertion of about 220 kbp is present in RGP29, which probably resulted from successive acquisition of two elements using the same chromosomal integration site. The RGP41-insertion in strain PA7 also provides hints for a combination of genome islands. Next to the pKLC102-like island with all typically conserved genes a DNA block with a second copy of some of the backbone genes is located, resembling a fragment of a second pKLC102-like element linked to the first one (Klockgether et al., 2008; Roy et al., 2010).

REPLACEMENT ISLANDS

Table 2 also lists the loci in the core genome that are under diversifying selection, the so-called replacement islands: RGP9 (flagellin glycosylation genes), RGP31 (O-antigen biosynthesis genes), RGP60 (pilin gene), and RGP73 (pyoverdine gene cluster). The RGPs only encompass those genes that fulfill the definition of less than 70% nucleotide sequence identity between homologs and thus do not necessarily comprise the complete functional units (Mathee et al., 2008).

The types of each replacement island were identified by comparative sequencing of the respective gene clusters in *P. aeruginosa* strain collections. The 20 known O-antigen serotypes, for example, were assigned to 11 groups according to the criterion of more than 98% sequence identity in the major O-antigen biosynthesis gene cluster (Raymond et al., 2002).

RGP60, containing the *pilA* gene that encodes the major subunit for type IV attachment pili, was classified into groups I–V (Kus et al., 2004). This "major pilin" region adjacent to a $tRNA^{Thr}$ gene contains, besides *pilA* for all groups but group II, several *tfp* genes that are involved in type IV pilus assembly and modification. More *tfp* genes are located downstream in the "minor pilin" region. Each of the five major pilin regions is associated with a specific set of minor pilins, and unrelated strains with the same major pilin type have identical minor pilin genes (Giltner et al., 2011). The absolute linkage disequilibrium between major and minor pilin groups provides evidence that both regions were derived from one large island. Consistent with this interpretation more pilin assembly genes are located between the major and minor pilin groups. These genes, however, were not subject of diversifying selection. Moreover a tRNA gene cluster is located between the major and the minor pilin region that serves as a hotspot for integration of large pKLC102-like islands (RGP41). Thus, the genome distance between major and minor pilin gene clusters varies between 136 kbp in strain PA14 and only 29 kbp in PAO1.

The pyoverdine gene clusters I, II, and III encode the three pyoverdine types and their specific receptor. Intratype divergence driven by recombination, positive selection, and horizontal gene transfer have enhanced the diversity of this genomic region (Smith et al., 2005).

The two flagellins a and b differ in their primary amino acid sequence and their glycosylation from each other (Spangenberg et al., 1996). b-type flagellins are conserved in sequence and glycosylation (Verma et al., 2006). In contrast, six *fliC* single nucleotide substitutions (SNPs) haplotypes (Spangenberg et al., 1996) and

differential glycosylation patterns lead to a large diversity of a-type flagellins (Arora et al., 2004). The variability of the a-type glycosylation gene cluster (RGP9) is high, even within the subtypes A1 and A2 that were defined by phylogenetic relatedness of amino acid sequences.

THE *P. AERUGINOSA* PANGENOME

The pangenome represents the complete gene pool of a bacterial species. Thus the description of a pangenome depends on the amount of sequence data available. For species with an extended accessory genome like *P. aeruginosa*, the addition of each new genome sequence will enlarge the overall pool of genes. The size of the core genome that is present in all strains will decrease concurrently.

To define the core genome and pangenome, the genomes are sequentially screened for orthologs by searching for reciprocal best BLAST hits. Genes that lack an ortholog in the already investigated gene pool are added to the pangenome.

We used the tool "Comparative Genome Search" provided by the *Pseudomonas* Genome Database[2] to define the number of orthologs representing reciprocal best blast hits in the four fully sequenced genomes of PAO1, PA14, LESB58, and PA7 (BLASTP comparisons, *E*-value cutoff: 1×10^{-4}). The tool also allows the determination of individual genes per genome, so the number of genes contributing to the pangenome could be counted with paralogs excluded. The results are shown in **Figure 5**. Please note that the PAO1 gene pool is lower than the overall number of ORFs in this genome (5520 compared to 5570) due to this exclusion of paralogs. As expected the core genome decreases and the pangenome increases each by a few hundred genes with the addition of a new genome. Although the analysis of just four genomes is insufficient for the extrapolation of the gene pool of core genome and pangenome of *P. aeruginosa*, we can assume that the pangenome does not approach a saturation value. Each novel genome sequence will contribute a yet unknown gene set to the pangenome. The large genomic islands of the pKLC102/PAGI-2 family contribute a broad variety of cargo to the species. Each strain possesses an individual set of islands that is acquired by horizontal gene transfer preferentially from beta- and gamma-proteobacteria (Klockgether et al., 2008). In other words, *P. aeruginosa* has wide, but not unrestricted access to the gene pool of prokaryotes.

INTRACLONAL GENOME DIVERSITY

The comparison of published genome sequences of clonally unrelated strains uncovered an interclonal sequence diversity of the *P. aeruginosa* core genome of 0.5–0.7% (Spencer et al., 2003; Cramer et al., 2011). The intraclonal diversity of members of the same clonal complex, however, is yet unknown. Of the strains with completely sequenced genomes, only strain PA14 belongs to a common clonal complex in the *P. aeruginosa* population (Wiehlmann et al., 2007). Hence we decided to sequence another strain of the PA14 clonal complex by Illumina sequencing-by-synthesis technology [study accession number ERP000390 at the Nucleotide Read Archive (ENA) of the EBI]. This strain RN3 was isolated from the first *P. aeruginosa*-positive airway specimen of an individual with cystic fibrosis who was living in North–West Germany. Strain PA14 is a clinical isolate from California. Thus the two strains are of unrelated geographic origin.

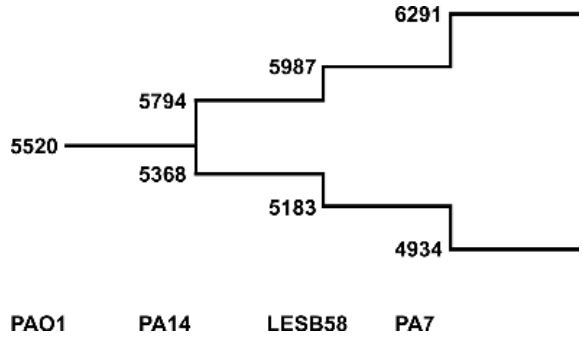

FIGURE 5 | The *P. aeruginosa* pangenome. The extent of the *P. aeruginosa* core- and pan-genome is shown as a stepwise development going along with the availability of complete genome sequences. The numbers at the lower branch give the amount of genes identified as best reciprocal blast hits in the indicated genomes (core genome). Numbers of the upper branch describe amount of genes making up the pangenome. For each genome the number of genes are added that are neither ortho- nor paralogs of genes from the existing pool.

The strain PA14 and strain RN3 genomes match in genome size and differ in 231 SNPs from each other (**Table 4**) which corresponds to a sequence diversity of 3.5×10^{-5}. Transitions ($n = 148$) occurred significantly more frequently than the expected ratio of transitions to transversions of 55: 176 of a random distribution ($\chi^2 = 206.3$; $P < 0.001$). The number of SNPs in inter- and intragenic regions roughly corresponded with their proportions in the genome. Within the coding regions synonymous SNPs were significantly overrepresented ($\chi^2 = 23.2$; $P < 0.001$) indicating that *de novo* amino acid substitutions had been subject to purifying selection.

Of the 231 SNPs, only 33 SNPs followed the statistics of a random distribution in the genome (**Figure 6**). In other words, 198 SNPs were non-randomly distributed in the genome implying that the affected loci had been subject to diversifying selection.

The major hotspot is the phage Pf1-like gene cluster (PA14_48890–PA14_49000) with 87 SNPs, i.e., 38% of all SNPs. Thus phage Pf1 seems to be the most rapidly evolving part of the PA14 genome consistent with the view that phages span a high degree of genetic diversity and are prone to frequent horizontal transfer (Hatfull, 2008).

Non-synonymous SNPs were mainly found in the functional categories of transcriptional regulators, membranes, cellular appendages, transport, and secretion (**Table 4**). Hotspots of sequence diversity in single genes between the PA14 and RN3 genomes are *ftsZ, armB (mexH)*, and *cynS* with six, five, and four SNPs, respectively. FtsZ is the major tubulin-like cytoskeletal protein in the bacterial cytokinesis machine (Erickson et al., 2010) and hence we noted with surprise that the FtsZ proteins of strains PA14 and RN3 differ at five positions in their amino acid sequence. The substitutions P-L, M-L, G-D, T-N, and P-T are located within a stretch of 35 amino acids of the 394 aa

[2]http://www.pseudomonas.com/geneSearch.jsp

Table 4 | Single nucleotide substitutions in RN3 sequence (compared to PA14 reference).

Intragenic position	nt	Locus_tag	aa	Gene name	Encoded product
72440	T – C	PA14_00740	K – E		Putative lipoprotein
96307	A – C	PA14_00970	syn.		Hypothetical protein
273734	C – T	PA14_03110	D – N		Hypothetical protein
477483	G – A	PA14_05410	syn.	*chpC*	putative chemotaxis protein
480880	T – C	PA14_05450	syn.		16S ribosomal RNA methyltransferase RsmE
480915	T – C	PA14_05450	K – E		16S ribosomal RNA methyltransferase RsmE
522777	G – T	PA14_05890	E – stop[1]		putative stomatin-like protein
741080	G – A	PA14_08660			$tRNA^{Gly}$
747764	G – A	PA14_08760	G – D	*rpoB*	DNA-directed RNA polymerase subunit beta
791890	A – G	PA14_09280	N – D	*pchF*	Pyochelin synthetase
888038[2]	C – T	PA14_10290	P – L	*acoR*	Transcriptional regulator AcoR
888039[2]	T – G	PA14_10290	P – L	*acoR*	Transcriptional regulator AcoR
927917	T – C	PA14_10770	I – T		Putative sensor/response regulator hybrid
982940	A – G	PA14_11290	syn.		Putative permease
1071133	G – C	PA14_12430[3]		*ladS*	Homolog to lost adherence sensor LadS
1082958	A – G	PA14_12630	syn.		Putative ATP-dependent helicase
1356548	G – C	PA14_15920	R – G	*yhjE*	Major facilitator transporter
1441164	T – C	PA14_16820	syn.		Putative efflux transmembrane protein
1468998	C – T	PA14_17130	syn.	*dxr*	1-deoxy-d-xylulose 5-phosphate reductoisomerase
1551564	G – A	PA14_18080	A – V		TetR family transcriptional regulator
1558205	A – G	PA14_18150	syn.	*acsL*	Putative acetyl-CoA synthetase
1612742	A –G	PA14_18740	syn.	*argG*	Argininosuccinate synthase
1640196	G – T	PA14_18985	P – H		Hypothetical protein
1640394	A – G	PA14_18985	F – S		Hypothetical protein
1880872	C – G	PA14_21690	A – G	*lhr1*	Putative ATP-dependent DNA helicase
1960256	C – A	PA14_22520	R – L		Hypothetical protein
2027678	C – G	PA14_23360	P – R	*wzz*	O-antigen chain length regulator
2149425	T – C	PA14_24600	syn.		Putative carboxypeptidase
2156146	C – A	PA14_24665	Q – K		Hypothetical protein
2209674	A – G	PA14_25250	K – E	*gapA*	Glyceraldehyde-3-phosphate dehydrogenase
2318606	A – G	PA14_26600	syn.		RNA polymerase sigma factor
2407435	C – G	PA14_27755	syn.	*yliJ*	Glutathione S-transferase
2407463	A – G	PA14_27755	K – E	*yliJ*	Glutathione S-transferase
2510099	A – G	PA14_29030	T – A		Putative FMN oxidoreductase
2545609	T – C	PA14_29390	syn.		Hypothetical protein
2545663	T – C	PA14_29390	syn.		Hypothetical protein
2553747	T – C	PA14_29440	D – G		LysR family transcriptional regulator
2651339	T – C	PA14_30600	F – L		Putative permease
2651357	A – G	PA14_30600	N – D		Putative permease
2762006	A – G	PA14_31750	K – E		Putative acyltransferase
2787777	C – G	PA14_32015[3]		*czcA*	Homolog to RND efflux transporter CzcA
2787784	T – G	PA14_32015[3]		*czcA*	Homolog to RND efflux transporter CzcA
2807266	G – C	PA14_32300	V – L		Putative kinase
2885933	G – C	PA14_32985	syn.	*gcvH2*	Glycine cleavage system protein H
2955357	A – G	PA14_33600	syn.		Hypothetical protein
2955433	A – G	PA14_33600	syn.		Hypothetical protein
2955468	A – G	PA14_33600	syn.		Hypothetical protein
2985345	A – G	PA14_33650	K – E	*pvdD*	Pyoverdine synthetase D
3198441	T – G	PA14_35940	syn.		Acyl-CoA synthetase
3373667	G – C	PA14_37830	syn.	*iscS*	Putative pyridoxal-phosphate dependent enzyme
3374601	A – G	PA14_37830	F – S	*iscS*	Putative pyridoxal-phosphate dependent enzyme

(Continued)

Table 4 | Continued

Intragenic position	nt	Locus_tag	aa	Gene name	Encoded product
3387854	A – C	PA14_37965	Y – S	*cynS*	Cyanate hydratase
3387881	A – C	PA14_37965	M – L	*cynS*	Cyanate hydratase
3387884	T – C	PA14_37965	F – L	*cynS*	Cyanate hydratase
3387941	A – C	PA14_37965	M – L	*cynS*	Cyanate hydratase
3390498	A – C	PA14_38000	Stop – S[4]		Hypothetical protein
3423281	A – G	PA14_38410	syn.	*amrB/mexH*	Multidrug efflux protein
3423414	C – G	PA14_38410	Q – E	*amrB/mexH*	Multidrug efflux protein
3424176	A – G	PA14_38410	T – A	*amrB/mexH*	Multidrug efflux protein
3424199	A – G	PA14_38410	syn.	*amrB/mexH*	Multidrug efflux protein
3425614	A – T	PA14_38410	H – S	*amrB/mexH*	Multidrug efflux protein
3442543	G – A	PA14_38580	G – D		Hypothetical protein
3443292	C – G	PA14_38580	P – A		Hypothetical protein
3541978	G – A	PA14_39750	syn.		Putative amino acid permease
3543662	A – G	PA14_39770	T – A		Putative regulatory protein
3558172	A – G	PA14_39910	F – L	*phzE2*	Phenazine biosynthesis protein PhzE
3559401	T – C	PA14_39925	K – E	phzD2	Phenazine biosynthesis protein PhzD
3566716	A – G	PA14_40020	Q – R		Hypothetical protein
3566730	A – G	PA14_40020	K – E		Hypothetical protein
3566749	A – G	PA14_40020	Q – R		Hypothetical protein
3566751	A – G	PA14_40020	N – D		Hypothetical protein
3566769	A – G	PA14_40020	K – E		Hypothetical protein
3566788	A – G	PA14_40020	Q – R		Hypothetical protein
3670384	A – G	PA14_41150	syn.		Putative permease of ABC transporter
3711749	T – C	PA14_41563	syn.	*cobA*	Uroporphyrin-III C-methyltransferase
3711791	G – C	PA14_41563	V – L	*cobA*	Uroporphyrin-III C-methyltransferase
3764383	A – G	PA14_42220	I – M		Membrane sensor domain-containing protein
3769180	C – G	PA14_42250	syn.	*pscL*	Type III secretion system protein
3879553	A – G	PA14_43570	F – L		Hypothetical protein
3906764	G – C	PA14_43870	R – G		Hypothetical protein
3933352	C – G	PA14_44190	syn.		Putative sugar MFS transporter
4346242	C – G	PA14_48890	syn.		Hypothetical protein
4346254	G – A	PA14_48890	syn.		Hypothetical protein
4346325	A – G	PA14_48890	syn.		Hypothetical protein
4346329	G – A	PA14_48890	syn.		Hypothetical protein
4346413	G – A	PA14_48890	syn.		Hypothetical protein
4346434	G – A	PA14_48890	syn.		Hypothetical protein
4346485	A – G	PA14_48890	syn.		Hypothetical protein
4346497	G – A	PA14_48890	syn.		Hypothetical protein
4346500	G – A	PA14_48890	syn.		Hypothetical protein
4346665	C – T	PA14_48890	syn.		Hypothetical protein
4346713	C – T	PA14_48890	syn.		Hypothetical protein
4346731	T – C	PA14_48890	syn.		Hypothetical protein
4346763	A – G	PA14_48890	syn.		Hypothetical protein
4346845	A – G	PA14_48890	syn.		Hypothetical protein
4346890	A – C	PA14_48890	syn.		Hypothetical protein
4346926	G – A	PA14_48890	syn.		Hypothetical protein
4346938	C – T	PA14_48890	syn.		Hypothetical protein
4347034	C – T	PA14_48890	syn.		Hypothetical protein
4347190	A – G	PA14_48890	syn.		Hypothetical protein
4347211	G – A	PA14_48890	syn.		Hypothetical protein
4347241	G – A	PA14_48890	syn.		Hypothetical protein

(Continued)

Table 4 | Continued

Intragenic position	nt	Locus_tag	aa	Gene name	Encoded product
4347256	C – T	PA14_48890	syn.		Hypothetical protein
4347283	T – C	PA14_48890	syn.		Hypothetical protein
4347289	G – C	PA14_48890	syn.		Hypothetical protein
4347310	T – C	PA14_48890	syn.		Hypothetical protein
4347322	T – C	PA14_48890	syn.		Hypothetical protein
4347346	C – G	PA14_48890	syn.		Hypothetical protein
4347358	G – A	PA14_48890	syn.		Hypothetical protein
4347376	C – G	PA14_48890	syn.		Hypothetical protein
4347642	G – A	PA14_48900	A – V		Hypothetical protein
4347673	T – A	PA14_48900	T – S		Hypothetical protein
4347701	C – A	PA14_48900	syn.		Hypothetical protein
4347825	G – T	PA14_48910	P – T		Hypothetical protein
4348119	C – T	PA14_48910	A – T		Hypothetical protein
4348192	A – G	PA14_48910	syn.		Hypothetical protein
4348221	G – A	PA14_48910	P – S		Hypothetical protein
4348224	T – C	PA14_48910	T – A		Hypothetical protein
4348308	G – A	PA14_48910	syn.		Hypothetical protein
4348378	G – T	PA14_48910	syn.		Hypothetical protein
4348501	A – G	PA14_48910	syn.		Hypothetical protein
4348684	A – G	PA14_48910	syn.		Hypothetical protein
4348966	T – G	PA14_48910	syn.		Hypothetical protein
4349128	A – C	PA14_48920	syn.		Bacteriophage protein
4350200	A – T	PA14_48930	syn.		Putative coat protein A of bacteriophage Pf1
4350213	G – C	PA14_48930	A – G		Putative coat protein A of bacteriophage Pf1
4350484	T – C	PA14_48930	N – D		Putative coat protein A of bacteriophage Pf1
4350502	T – C	PA14_48930	T – A		Putative coat protein A of bacteriophage Pf1
4350656	G – A	PA14_48930	syn.		Putative coat protein A of bacteriophage Pf1
4350884	A – G	PA14_48940	syn.	*coaB*	Coat protein B of bacteriophage Pf1
4350911	G – C	PA14_48940	syn.	*coaB*	Coat protein B of bacteriophage Pf1
4350917	A – G	PA14_48940	syn.	*coaB*	Coat protein B of bacteriophage Pf1
4350941	A – G	PA14_48940	syn.	*coaB*	Coat protein B of bacteriophage Pf1
4350959	T – C	PA14_48940	syn.	*coaB*	Coat protein B of bacteriophage Pf1
4351186	C – T	PA14_48950	A – T		Hypothetical protein
4351199	G – A	PA14_48950	syn.		Hypothetical protein
4351316	A – G	PA14_48950	syn.		Hypothetical protein
4351503	G – A	PA14_48970	syn.		Helix destabilizing protein of bacteriophage Pf1
4351563	T – C	PA14_48970	syn.		Helix destabilizing protein of bacteriophage Pf1
4351617	A – G	PA14_48970	syn.		Helix destabilizing protein of bacteriophage Pf1
4351641	C – G	PA14_48970	syn.		Helix destabilizing protein of bacteriophage Pf1
4351722	A – G	PA14_48970	syn.		Helix destabilizing protein of bacteriophage Pf1
4351857	G – A	PA14_48970	syn.		Helix destabilizing protein of bacteriophage Pf1
4352075	A – G	PA14_48980	syn.		Hypothetical protein
4352106	T – C	PA14_48980	D – G		Hypothetical protein
4352113	C – A	PA14_48980	D – Y		Hypothetical protein
4352144	G – C	PA14_48980	S – R		Hypothetical protein
4352234	G – A	PA14_48980	syn.		Hypothetical protein
4352294	G – C	PA14_48980	syn.		Hypothetical protein
4352384	C – T	PA14_48980	syn.		Hypothetical protein
4352465	G – T	PA14_48990	syn.		Hypothetical protein
4352471	G – A	PA14_48990	syn.		Hypothetical protein
4352545	C – A	PA14_48990	A – S		Hypothetical protein

(Continued)

Table 4 | Continued

Intragenic position	nt	Locus_tag	aa	Gene name	Encoded product
4352560	G – A	PA14_48990	P – S		Hypothetical protein
4352594	C – G	PA14_48990	syn.		Hypothetical protein
4352607	C – T	PA14_48990	R – Q		Hypothetical protein
4352676	G – C	PA14_48990	A – G		Hypothetical protein
4352700	T – C	PA14_48990	H – R		Hypothetical protein
4352821	T – C	PA14_49000	I – V		Hypothetical protein
4352865[2]	A – C	PA14_49000	I – G		Hypothetical protein
4352866[2]	T – C	PA14_49000	I – G		Hypothetical protein
4352921	C – A	PA14_49000	M – I		Hypothetical protein
4450619	T – G	PA14_50060	L – R		Hypothetical protein
4565005	C – G	PA14_51360	G – A	*phnA*	Hnthranilate synthase component I
4565040	A – G	PA14_51360	syn.	*phnA*	Hnthranilate synthase component I
4565093	C – G	PA14_51360	G – R	*phnA*	Hnthranilate synthase component I
4707658	G – C	PA14_53110	syn.		Hxidoreductase
4707787	G – C	PA14_53110	syn.		Oxidoreductase
4760743	A – G	PA14_53670	L – P		Hypothetical protein
4901696	A – G	PA14_55180	M – V	*migA*	Glycosyl transferase
4912690	C – T	PA14_55330	D – N		Hypothetical protein
4947683	A – G	PA14_55600	H – R		Hypothetical protein
4997786	T – C	PA14_55980	K – E	*yjgR*	Hypothetical protein
5041775	A – T	PA14_56550	syn.		Hypothetical protein
5103224[2]	A – C	PA14_57275	P – L	*ftsZ*	Cell division protein FtsZ
5103225[2]	G – A	PA14_57275	P – L	*ftsZ*	Cell division protein FtsZ
5103259	T – G	PA14_57275	M – L	*ftsZ*	Cell division protein FtsZ
5103291	C – T	PA14_57275	G – D	*ftsZ*	Cell division protein FtsZ
5103303	G – T	PA14_57275	T – N	*ftsZ*	Cell division protein FtsZ
5103322	G – T	PA14_57275	P – T	*ftsZ*	Cell division protein FtsZ
5236534	A – G	PA14_58760	syn.	*pilC*	Type 4 fimbrial biogenesis protein pilC
5404627	C – T	PA14_60630	L – F		Hypothetical protein
5464577	C – T	PA14_61200	G – D		Hypothetical protein
5530315	A – G	PA14_62000	F – L	*hitA*	Ferric iron-binding periplasmic protein HitA
5722900	A – C	PA14_64230	D – A	*retS/rtsM*	RetS, regulator of exopolysaccharide and type III Secretion
5757525	G – C	PA14_64620	Q – E		Putative oxidoreductase
5757527	G – C	PA14_64620	P – R		Putative oxidoreductase
5809365	T – C	PA14_65190	K – E	*yjfH*	TrmH family RNA methyltransferase, group 3
5866730	T – G	PA14_65860	syn.		Putative two-component sensor
5905079	A – G	PA14_66270	syn.	*glnE*	Glutamine-synthetase adenylyltransferase
5968025	C – G	PA14_66820	P – A	*phaC1*	Poly(3-hydroxyalkanoic acid) synthase 1
6070122	T – C	PA14_68020[3]			Homolog to hypothetical protein PA5149
6076066	G – C	PA14_68100	syn.		Hypothetical protein
6412470	G – C	PA14_71930	R – G	*wbpX*	Glycosyltransferase WbpX
6441338	T – C	PA14_72300	L – P		Hypothetical protein

Intergenic position	nt	Intergenic region
151966	A – G	igrPA14_01660-01670
187759	G – T	igrPA14_02050-02060
208430	T – G	igrPA14_02310-02330
208433	G – C	igrPA14_02310-02330
888497	A – C	igrPA14_10290-10300
966217	T – C	igrPA14_11110-11120
1144646	C – G	igrPA14_13320-13330

(Continued)

Table 4 | Continued

Intergenic position	nt	Intergenic region
1375947	A – G	igrPA14_16150-16160
1725505	A – G	igrPA14_20020-20030
1748240	T – C	igrPA14_20290-20300
1923008	C – T	igrPA14_22080-22090
2354149	A – G	igrPA14_27090-27100
2362330	A – C	igrPA14_27180-27190
2362363	G – C	igrPA14_27180-27190
2589402	C – T	igrPA14_29890-29900
2840442	T – C	igrPA14_32700-32710
2840444	T – C	igrPA14_32700-32710
3281477	G – C	igrPA14_36810-36820
3356495	G – C	igrPA14_37680-37690
3515863	T – C	igrPA14_39480-39500
3662614	T – C	igrPA14_41070-41080
4347602	G – C	igrPA14_48890-48900
4351470	T – G	igrPA14_48960-48970
4352019	T – G	igrPA14_48970-48980
4352023	C – G	igrPA14_48970-48980
4352432	G – A	igrPA14_48980-48990
4352433	G – A	igrPA14_48980-48990
4407236	A – G	igrPA14_49540-49560
4659805	A – G	igrPA14_52530-52540
4708161	A – G	igrPA14_53110-53120
5198405	T – C	igrPA14_58360-58375
5200474	A – C	igrPA14_58380-58390
5565118	A – G	igrPA14_62380-62390
5648548	A – G	igrPA14_63280-63290
5648573	A – G	igrPA14_63280-63290
5792010	A – G	igrPA14_64980-64990

[1] *Protein length 180 aa instead of 264.*

[2] *Two SNPs in one codon.*

[3] *Annotated as probably inactive protein fragment/putative frameshift gene.*

[4] *Next stop 18 codons downstream.*

protein and are all not neutral (**Table 4**). MexH is a component of the MexGHI-OpmD efflux pump that is required for biofilm formation (Southey-Pillig et al., 2005), facilitates cell-to-cell communication and promotes virulence and growth in *P. aeruginosa* (Aendekerk et al., 2005). MexH of strains PA14 and RN3 differ by three amino acid substitutions (Q-E, T-A, and H-S) in three distant domains of the protein from each other. *CynS* encodes a cyanase (EC 4.2.1.104) that catalyzes the decomposition of cyanate into CO_2 and ammonium (Luque-Almagro et al., 2008). The intraclonal diversity of cyanase between RN3 and PA14 of four amino acid substitutions is similar in number and localization to that of the completely sequenced *P. aeruginosa* strains, i.e., 5–11 amino acid substitutions clustering in the N-terminal region of CynS.

Key genes were also affected by non-synonymous SNPs that may modulate the function of the gene products. The DNA-directed RNA polymerase RpoB of strain RN3 carries a substitution of a glycine by an aspartate, and the global regulator RetS of the sessile and planktonic lifestyle of *P. aeruginosa*, which is involved in the transition from acute to chronic infections (Goodman et al., 2004), harbors a substitution of an aspartate by an alanine.

Of the 34 observed amino acid substitution types, nine are classified by the Dayhoff (1978) matrix as uncommon and associated with an impact on protein function. In contrast, only 12 of the 20 most common neutral amino acid changes were seen. In summary, SNPs non-randomly targeted elements of the cell surface and uncommon non-neutral substitutions (e.g., K-E) were overrepresented in the affected proteins. These facts suggest that in the investigated case the intraclonal diversity did not evolve by random drift, but was driven by selective forces.

Strain RN3 was isolated from the first *P. aeruginosa*-positive specimen taken from an individual with cystic fibrosis. Thus the portion of adaptive mutations that typically emerge during chronic colonization of cystic fibrosis airways (Smith et al., 2006) should be low. Nevertheless some sequence differences between RN3 and PA14 could provide RN3 with

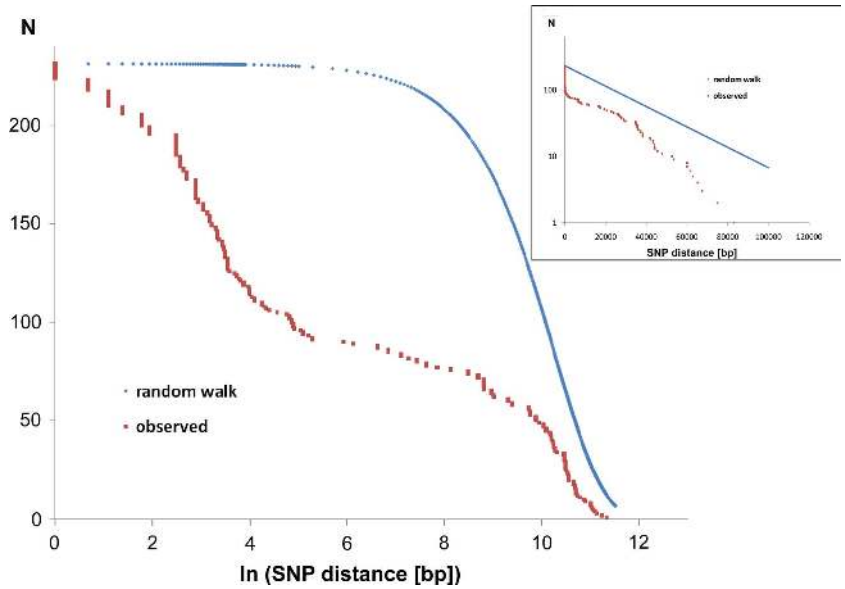

FIGURE 6 | Intraclonal SNP diversity of the *P. aeruginosa* PA14 clone: distribution of nearest SNP neighbors in the RN3 genome. Mapping of the RN3 genome onto the PA14 genome uncovered 231 SNPs. The figure depicts the genomic distribution of the distance between two adjacent SNPs (nearest neighbors). The red graphs show the observed distribution that is compared with a random genomic distribution of the same number of 231 SNPs (blue graphs, one-dimensional random walk statistics). The two semilogarithmic plots visualize the deviation from a random distribution at either a global scale (insert) or with focus on the hotspots of sequence diversity (large figure).

selective advantage to adapt and persist in cystic fibrosis airways. Obvious candidates are loci encoding efflux pumps (*mexH*), major transcriptional regulators (*retS*), and siderophore (*pvdD*), cyanide (*cynS*), or quinolone (*phnA*) biosynthesis, respectively.

The major take home message of our endeavor to compare the intraclonal genome diversity of strains of distant geographic origin was the unexpectedly low substitution rate. Statistical analysis provides strong evidence that nucleotide substitutions in coding regions were under purifying selection so that only a low number of substitutions was fixed. This versatile, ubiquitous and phylogenetically ancient organism apparently does not need many *de novo* mutations if it conquers a new habitat. The next step to understand the molecular evolution of intraclonal diversity would be the determination of the relative contributions of *de novo* mutation versus recombination. To accomplish this task, a larger collection of clone PA14 strains than just two isolates will have to be studied (see Spratt, 2004, for an appropriate study design).

PERSPECTIVES

Only four completely sequenced *P. aeruginosa* genomes are officially deposited as finished genomes in GenBank. Draft genomes exist for a five further genomes and several dozen *P. aeruginosa* projects are deposited in the ENA hosted by EMBL-EBI (see text footnote 1). Many of the projects were done for the purpose of (re)sequencing variants of already known strains. Thorough genome assemblies and functional annotations are probably intended only in a minority of cases. But nevertheless an immense increase in *P. aeruginosa* genome data is expected to become available in the near future due to the on-going revolution of sequencing technologies. In particular, the sequencing of strains from environmental habitats should provide us with an unbiased overview of the genetic repertoire of the *P. aeruginosa* population.

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