Exploring the success of Brazilian endemic clone *Pseudomonas aeruginosa* ST277 and its association with the CRISPR-Cas system type I-C

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Abstract

Background The Brazilian endemic clone *Pseudomonas aeruginosa* ST277 carry important antibiotic resistance determinants, highlighting the gene coding for SPM-1 carbapenamase. However, the resistance and persistence of this clone is apparently restricted to Brazilian territory. To completely understand the differences between strains from Brazil from the ones isolated in other countries, we phylogenetically examined 43 *P. aeruginosa* ST277 genomes as well as analyzed virulence and resistance genes, evaluated the distribution of genomic islands and assessed in detail the characteristics of the CRISPR-Cas immunity system in these isolates. **Results** The Brazilian genomes presented a typical set of resistance and virulence determinants, genomic islands and a high frequency of the CRISPR-Cas system type I-C. Upon phylogeny results, even if ST277 genomes are closely related we can see important differences among the strains isolated from China, Mexico and United States when compared to others, most of them from Brazil. We also observed a standard CRISPR spacers content for ST277, confirming a strong link between phylogeny and spacer acquisition. **Conclusions** Based on our findings, *Pseudomonas aeruginosa* ST277 strains circulating in Brazil have acquired a few distinctive genetic elements that contributing to their resistance, pathogenicity and success when compared to strains from other countries.

Introduction

Pseudomonas aeruginosa is an important pathogen frequently implicated in healthcare-associated infections having a strong potential for development of multidrug resistance. Since the first report in 2002, SPM-1 metallo- β -lactamase is the main carbapenem resistance mechanism associated with *P. aeruginosa* in Brazil [1, 2]. To date, the *bla*_{SPM-1} is restricted to *P. aeruginosa* and there are only two confirmed cases outside of Brazil, both of which received medical treatment while in this country [3, 4]. Although SPM-1-producing *P. aeruginosa* has been mainly isolated from nosocomial settings, reports of this multidrug-resistant bacterium in urban rivers and microbiota of migratory birds in Brazil alert to the dispersion of this important resistance mechanism [5, 6]. Usually, the *bla*_{SPM-1} gene is inserted in the mobile genetic element ICE_{Tn4371}6061 [7] and in turn, this ICE is located in *P. aeruginosa* Genomic Island 15 (PAGI-15) [8].

SPM-1-producing *P. aeruginosa* descends from a common ancestor, a clone belonging to the ST277 [2]. This clone has been characterized as a resistance-enriched ST [9], and the expression of SPM-1 generates resistance to all β -lactams, except for aztreonam [8]. Besides SPM-1, other genetic determinants have been associated with ST277: i) the class 1 integron *ln163* carrying three resistance genes (*aacA4*, *bla*_{OXA-56} and *aadA7*); ii) *rmrD* gene that confers high-level resistance to most aminoglycosides; and iii) the type I-C of Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) and associated proteins [9–11].

The CRISPR family of repetitive DNA sequences, together with a group of CRISPR-associated (*cas*) genes, were described by Jansen and coworkers in 2002 [12]. The CRISPR-Cas system encodes a unique

defense mechanism that acts against invading genetic elements such as viruses and plasmids. CRISPR loci consist of an array of short and partially palindromic, repetitive sequences interspaced by equally short 'spacer' sequences from viral or plasmid origin. In the immunization stage, spacer sequences are captured and integrated into the first position of the CRISPR array. In the immunity stage, the spacer guides de Cas nucleases to target invading DNA carrying a corresponding sequence which is cleaved [13]. The spacer sequences define the specificity of the CRISPR–Cas immune response and store the memory of past encounters with foreign DNA [14].

The *cas* genes are diverse and frequently suffer modular recombination which resulted in the currently adopted classification scheme that partitions the CRISPR-Cas systems into two distinct classes. The Class 1 system is widespread among Archaea and Bacteria, and includes type I, type III, as well as the rare type IV. The *cas8c* gene is the signature gene for subtype I-C which includes others six *cas* genes [15]. *P. aeruginosa* has emerged as a major CRISPR-Cas model system. Types I-F and I-E are the CRISPR-Cas system most commonly found in this specie [9].

Here, we examine the phylogenetic distribution and conservation of genetic determinants among 43 ST277 *P. aeruginosa* genomes available at NCBI. We aim to provide depth evidence about the characteristics that have contributed for resistance and persistence characteristics of this clone, only described for Brazilian associated isolates.

Results

We compared the sequences of 43 strains to understand the genomic diversity of *P. aeruginosa* ST277. According to their BioSample records, strains from a 20-year period were included in this study (1997–2017). The vast majority obtained were from Brazil (33/43), and overall, they represent human-derived isolates (31/43). The other countries represented are United States (6), United Kingdom (1), Mexico (1) and China (1). Phylogenetic reconstruction based on SNPs showed that the ST277 genomes (disregarding PAO1) shared a total number of 16,649 SNPs (4,517 core and 12,132 non-core). The clade including all ST277 genomes share 510 unique SNPs, while the clade with genomes from Brazil, 4 from United States and the single from United Kingdom share 920 unique SNPs. Overall, the genomes' phylogenetic relationships do not seem to be associated to the year of isolation (Figure 1).

Fig. 1: Whole-genome SNP-based parsimony tree of 43 ST277 *P. aeruginosa* isolates and the reference genome PAO1 generated by kSNP3.0. The branch lengths are expressed in terms of changes per number of SNPs. After strains identification, separated by an underline, there are the SNP counts. The tree was visualized using Dendroscope (40). The paint shows the presence (black), absence (gray) or duplication (blue) of the genetic determinants surveyed. The purple bars represent an additional mutation in the *gyrA* gene (D87N), while the green bars represent samples with spacers organization other than ST277 standard spacers content. BR: Brazil, CH: China, MX: Mexico, USA: United States of America, UK: United Kingdom, Hum: Human, Env: Environmental, -: not available.

We computationally searched for resistance genes in all ST277 strains. These data demonstrate that 90% (39/43) of the genomes are positive for *ln163*, 70% (30/43) for *bla_{SPM-1}* (carbapenem resistance), 65% (28/43) for *rmtD* (aminoglycoside resistance), and 42% (18/43) for *crpP* (ciprofloxacin resistance). All ST277 strains carry the genes *bla_{OXA-50}* (beta-lactam resistance), PDC-5 (beta-lactam resistance, *Pseudomonas*-derived cephalosporinases), *catB7* (chloramphenicol resistance), *bcr1* (bicyclomycin resistance), *fosA* (fosfomycin resistance) and *aph(3')-IIb* (aminoglycoside resistance). The prevalence of OXA-50, PDC-5, *catB7*, *bcr1*, *fosA* and *aph(3')-IIb*, among 2,576 *P. aeruginosa* whole-genome shotgun assemblies available at NCBI, are higher than 97% [16]. Therefore, these genes are related to the *P. aeruginosa* specie, and not exclusively to the ST277 (Figure 1, Additional file 1).

Non-synonymous mutations were found in the following resistance related genes: *oprD*, *mexT*, *gyrA*, *nalC*, *pmrA*, and *phoQ*. All ST277 strains have the same amino acid alterations in OprD (T103S, K115T), NalC (S209R, G71E), and PmrA (L71R), besides 8 nucleotide deletion on *mexT* (240–247), which caused frameshift. Also, 38 strains (88%) have 2 nucleotide deletion on *oprD* (379–380) and 19 bp deletion on *mexZ* (437–456) genes which caused [the] frameshift. Forty strains have the T83I mutation on GyrA in addition to D87N mutation on the same protein observed for 9 strains (21%); and one strain has a mutation on PhoQ (V260G) (Figure 1, Additional file 1). Together, the mechanisms found in this work that may contribute to antimicrobial resistance in the *P. aeruginosa* samples of ST277 are shown in Table 1.

Table 1: Mechanisms contributing to antimicrobial resistance in the ST277 strains.

Mutations in the virulence genes were analyzed using the genes from *P. aeruginosa* PAO1 as a reference. Fourteen strains (32.5%) have different point mutations or one nucleotide deletion in *lasR* gene, 7 strains (16%) have the same non-synonymous mutation on *rhIR* (K222T), and a fragment with eleven nucleotides was absent in *algB* (382–393) for 3 strains (7%). All ST277 strains carrying the virulence genes *exoS*, *exoT* and *exoY* (Figure 1, Additional file 1).

Genomic comparisons using pangenome as a reference visually showed that some regions are not shared by all ST277 genomes. We verified that these regions were PAGIs, already described in previous works (PAGI-13, PAGI-14, PAGI-15, PAGI-19, PAGI-23, PAGI-25, PAGI-29, PAGI-32, PAGI-34, PAGI-36 and PAGI-39) [8, 17], or a plasmid with 49 Kb, present in 3 strains (Additional file 8). Furthermore, some of these PAGI harbor typical ST277 genetic determinants. In case of strains positive for the *bla_{SPM-1}* gene, it is inserted inside PAGI-15. For those strains that carrying class 1 integron *ln163* or *rmtD* gene, they are contained in PAGI-25.

Computational analysis identified intact type I-C CRISPR-Cas system in 30 genomes from ST277 (70%) (Figure 1, Additional file 1). Two *P. aeruginosa* isolates (AZPAE14819 and AZPAE14822) have the complete Cas operon, but the CRISPR associated was not detected. No other type of CRISPR-Cas system

was found among the ST277 strains. When present, the type I-C CRISPR-Cas system was uniformly localized on PAGI-34 in the ST277 genomes analyzed here.

We analyzed the whole spacers content present in type I-C CRISPR-Cas system identified in *P. aeruginosa* genomes. Among ST277 strains with intact type I-C CRISPR-Cas system, 90% (27/30) have the same 39 spacers content, considered by us the standard spacers content of this ST (Additional file 2). The strain 213BR has a CRISPR array of 22 and another of 11 spacers, separated by 200bp from each other and near to Cas operon. These 33 spacers are included in the ST277 standard spacers content. We detected three CRISPR arrays in the strain PA19, with 12, 13 and 6 spacers, all of them included in the ST277 standard spacers content. The first CRISPR array (12 spacers) is associated with Cas operon. Strain LIM1680 has a set of 37 spacers, associated with Cas operon and included in the ST277 standard spacers content. We noticed that 64% (25/39) of the spacers are present in all genomes from this ST suggesting a strong link between phylogeny and spacer content. We need to consider that possible sequencing and assembly errors may have influenced the spacers identification, so the percentage can be higher than 64%. The ST277 spacers at both ends are conserved between the strains, and the deleted/unidentified spacers are positioned in CRISPR central region (Figure 2).

Fig. 2: Spacer content among *P. aeruginosa* carrying type I-C CRISPR-Cas system. The numbered spacers belong to the ST277 standard spacers content. Colored and numbered spacers are those shared between four different clones (ST277, 235, 17 and 853).

From 57 NCBI genomes non-ST277 which have type I-C CRISPR-Cas system, 6 (10%) are from ST235. The other 51 genomes are from at least 32 different STs (Additional file 1). All isolates with type I-C CRISPR-Cas system appear to harbor PAGI-34, but lack some regions. The clones with the PAGI-34 more like to that of the ST277 belong to STs 17, 235, 853, and 1232 (Additional file 9).

The CRISPR spacers associated with Cas subtype I-C were analyzed, and a total of 345 non-redundant spacers were obtained (Additional file 2). Most non-ST277 genomes belonging to ST235 (5/6) have 21 spacers, and 80% of them are corresponding to the spacers in the ST277 standard spacers content. Besides ST235, two other STs have spacers that match those of the ST277 standard spacers content (ST17 and ST853). Eleven spacers were identified in the genomes from these four clones (spacers 3, 4, 5, 28, 29, 30, 32, 33, 37, 38 and 39). Most of them belong to the CRISPR trailer end (Figure 2).

Besides type I-C CRISPR-Cas subtype, 29 genomes also have type I-F CRISPR-Cas subtype. These genomes are from 18 different STs (17, 160, 274, 439, 553, 557, 589, 645, 646, 874, 1074, 1184, 1527, 1605, 1920, 2329, 2651 and 2717) (Additional file 1).

BLAT between the ST277 standard spacers content and the NCBI plasmid and phage databases assigned targets for 8% (3/39) and 38% (15/39) of the unique spacers, respectively. In some cases, a unique spacer presented homology with more than one phage and plasmid. We observed that the spacers with the greatest number of matches are distributed throughout the CRISPR array (Figure 3, Additional file 3).

Fig. 3: Number of ST277 spacers' matches against public sequences of plasmid and phage. X: Spacers; Y: Number of matches.

Among the available plasmids and phages in the NCBI, 6 plasmids and 40 phages were targeted by some spacer (Additional file 3). Two target plasmids harbor an integron encoding resistance to aminoglycosides, beta-lactam, and sulfonamides (pPB354_1 and pPB353_1). Among the phages, 15 perform a lysogenic cycle, 2 a lytic cycle, 2 lytic-lysogenic switch, and for 21 we could not have this information.

We searched for anti-CRISPR genes (*acr*) in ST277 genomes, and on phage and plasmids targeted by CRISPR spacers for subtype I-C. Proteins were identified as candidate anti-CRISPRs in 4 *P. aeruginosa* genomes (Additional file 5). Among these genomes, two have CRISPR-Cas system (2/30) but they are not present in the other two. In total, 3 plasmids (3/6) and 20 phages (20/40) have putative Acr proteins (Additional file 6–7, respectively). The two plasmids harboring resistance genes described above also code for putative Acr proteins. Between the 27 anti-CRISPRs candidates' genes found in this study, 63% were identified as known groups of *acr* genes, all them characterized as anti-type I-F (Additional file 4).

Discussion

P. aeruginosa ST277 is a clone spread throughout Brazilian territory and related to high mortality rates [2, 10, 18]. It has been reported that *In163*, *rmtD* and *bla*_{SPM-1} are frequently associated to this clone [2, 10, 17, 18]. *In163* carries three gene cassettes that confer resistance to aminoglycosides and cephalosporins; *aacA4*, *bla*_{OXA-56}, and *aadA7* [10]. The rRNA methylase RmtD promotes high-level resistance to all clinically available aminoglycosides [11]. The SPM-1 carbapenemase causes resistance to all β -lactam but aztreonam [1]. Together, these mechanisms cause resistance to the main antimicrobials used against *P. aeruginosa* in clinical practice.

SPM-1 producing *P. aeruginosa* is almost exclusively reported in Brazil, mostly on ST277 [2]. There are only two reports out of Brazil, in patients who received medical care while traveling to Brazil, one from UK and the other from Switzerland [3, 4]. Analyzing the available genomes in the Genbank, we identified an ST277 genome from UK, however we could not identify *bla*_{SPM-1} in it (NCTC13921, NZ_UAUD00000000). On the other hand, we identified the *bla*_{SPM-1} gene in a USA strain isolated in 2005 (WHSGIV07484, NZ_LLPQ00000000), but there is no official description about this fact.

According to our observations, the arsenal of acquired antibiotic resistant genes characteristic of ST277 seems relatively restricted and strains from this clone were mostly isolated in Brazil (77%). This scenario differs from other *P. aeruginosa* high-risk clones such as ST235, which shows a worldwide distribution (more than 20 countries) and possesses a great diversity of resistant genes including 9 β -lactamases families and 22 aminoglycosides transferases genes [19].

We also analyzed mutational events in some genes that can contribute to enhance bacterial resistance phenotype. Efflux pumps provide resistance to different antibiotics classes by extruding these molecules.

When efflux pumps are associated with other mechanisms, they have better effects on the change of the clinical strain classification from susceptible to intermediate / resistant [20]. MexAB-OprM efflux pump is negatively regulated by *nalC*. The NalC amino acid changes observed here have previously been described in isolates not displaying MexAB-OprM overexpression [20]. Thus, this mechanism is not contributing to resistance in the ST277 strains analyzed here.

The gene *mexT* is a MexEF-OprN efflux pump positive regulator. However, the wild type of *mexT* is inactive. Some mutation can drive to an active mode, known as *nfxC* mutant. The absence of 8bp in *mexT* gene detected here for all ST277 strains is a type of *nfxC* mutant and has been associated with decreased sensitivity to antibiotics such as chlorophenylcholine and fluoroquinolones, and increased virulence due to efflux of molecules from the quorum sense signaling system [21]. We suggest that *nfxC* mutant are an ST277 characteristic.

The MexXY-OprM system is one of the few capable of transporting aminoglycosides. This efflux pump also contributes to resistance to cefepime, tetracyclines and macrolides [22]. The sequence of repressor gene *mexZ* lost 19 bp in 88% of the ST277 strains. This deletion has already been related to overexpression of MexXY-OprM system [8].

Mutations in the PmrAB or PhoPQ two-component regulatory systems can mediate colistin resistance. A review of the literature indicated that the mutation in PmrA observed for all ST277 strains have previously been reported in colistin sensitive strains [23], while the PhoQ amino-acid alteration noticed here for only one strain (213BR) is associated with a colistin-resistant phenotype [24].

One of the main mechanisms of resistance to fluoroquinolones is the occurrence of site-specific mutations in the genes of the DNA gyrase (*gyrA* e *gyrB*). Both mutations found in this work for GyrA have been described in quinolone-resistant clinical isolates of *P. aeruginosa*. While the substitution of a threonine by an isoleucine at position 83 observed for 40 ST277 strains is detected frequently, the mutation D87N found for 9 strains is less ordinary [25].

The *oprD* frameshift observed in 88% of the ST277 strains causes an important reduction in the *oprD* transcriptional levels and affects porin functionality which is strongly associated imipenem resistance [8, 20]. We also identified 20 *oprD* mutations for all ST277 strains. Only two mutations are non-synonymous, but they are unable to affect the flow of molecules through this bacterial membrane' pore. The presence of a potential association has been suggested between specific amino acid substitutions in OprD and MLST profiles [26]. Perhaps the mutations observed here can be characteristic of ST277.

The protein AlgB is involved in the high-level production of alginate by *P. aeruginosa* which consequentially expresses a mucoid phenotype [27]. Deletion on this gene observed for some strains thereby can impact upon the strains virulence. *ExoS*, *exoT* and *exoY*, present in all ST277 genomes, encode type III secretion system' effector proteins. Most *P. aeruginosa* strains carry *exoT* and *exoY* genes while the presence of *exoS* and *exoU* appear to be mutually exclusive. *ExoS* positivity correlates strongly with the presence of multi-drug resistance but is less virulent than *exoU* strains [28].

P. aeruginosa quorum sensing (QS) constitutes a highly complex mechanism of bacteria cell-cell communication that plays a key role in modulating the expression of virulence genes and a wide range of metabolic functions. The *las* and *rhl* are two QS systems in *P. aeruginosa*, of which LasR and RhlR are cognate transcriptional regulators, respectively [29]. The spontaneous mutation on these genes observed here can affect gene function and cause bacteria' phenotypic and virulence alterations [29].

The presence of the type I-C CRISPR-Cas system was first reported in *P. aeruginosa* just recently, mainly associated do STs 277 e 235. This system is located at PAGI-34, a pKLC102-like ICE, the first putatively mobile genetic element with a CRISPR-Cas system in a *Pseudomonas* species [9].

In this study, 70% of ST277 genomes available at NCBI harbor type I-C CRISPR-Cas system. We also identified this system in other 57 genomes from at least 32 different STs. We believe that the association between type I-C and ST277 is strong when compared to other STs, but more analysis is needed to state this. Some of non-ST277 genomes harbor type I-F CRISPR-Cas system besides type I-C. Type I-F is the most commonly intact CRISPR-Cas system found in *P. aeruginosa* [9].

Belkum and coworkers identified positive correlations between the type I-C CRISPR-Cas subtype and the *rmfD*, *aadA7*, and OXA-encoding resistance genes [9]. As previously mentioned, the incidence of *In163* (harboring *aadA7* and *bla*_{OXA-56}) and *rmfD* is high in strains of clone ST277 [10, 17, 18]. We did not find these same genetic structures in other STs where type I-C CRISPR-Cas subtype was identified. It seems that *rmfD*, *aadA7*, and OXA-encoding resistance genes are correlated to ST277, not to the type I-C CRISPR-Cas system.

Aware of the evolutionary and immunological information provided by CRISPR spacers [14], we analyzed the whole spacers content present in type I-C CRISPR-Cas subtype identified in *P. aeruginosa* genomes. We observed a standard spacers content for ST277. This set varied a little for only 3 strains, but we believe this may be associated with problems in the NCBI genomes' sequencing and / or assembly. A strong link between phylogeny and spacer content in *P. aeruginosa* clinical isolates was already reported previously [9].

In regard to the spacers from other STs, only three clones share identical spacers with ST277 standard spacers content (ST235, 17 and 853). The PAGI-34 of these clones have the same genetic structure of ST277' PAGI-34. None of these STs share equal alleles for genes in the *P. aeruginosa* MLST scheme, suggesting that they are phylogenetically unrelated. The fact that the type I-C CRISPR-Cas system from these strains is inserted in very similar mobile genetic element could justify unrelated clones with the same spacers. Usually, the newest spacers are located at the CRISPR' leader end and the most ancestral spacer sequences are positioned at the trailer end [14]. Most of the spacers shared by the four STs belong to the CRISPR trailer end, representing the most ancestral invasions.

We also analyzed the phages and plasmids that were the possible spacers' targets. The rates of spacers with assigned targets were close to those found by Belkum and coworkers, who used the same

identification strategy [9]. Most spacers with matches originated from phage sequences, which are in accordance with the origin of the CRISPR-Cas system, primarily engaged in antiviral defense [30].

It has been described a trend in drop the fraction of spacers with matches from the beginning to the end of CRISPR arrays [30]. We found an irregular distribution of the spacers with the greatest number of matches throughout the CRISPR array. As we are studying a specific Cas subtype in a particular *P. aeruginosa* clone this may be influencing the uncommon distribution found here.

Strains with intact CRISPR-Cas systems can be phenotypically CRISPR-Cas incompetent if they encode a cognate anti-CRISPR protein (Acr) that deactivates the corresponding CRISPR-Cas system [9]. The anti-CRISPR-associated protein 1 (Aca1) is a highly conserved sequence located downstream to *acr* gene, the latter with variable sequences classified in different families [31, 32].

We did not find homologous to the unique anti-type I-C gene described until now (*acrIC1* from *Moraxella bovoculi*) [33]. However, all putative Acr proteins identified in this work, even those with anti-type I-F proven activity, should be tested for activity in strains of *P. aeruginosa* possessing type I-C system. The dual specificity of some anti-CRISPR proteins has already been described. AcrF6Pae can inactivate both type I-F and I-E CRISPR-Cas systems [31], while AcrVA3.1 may inhibit the type I-C as well as type V-A system [33].

Conclusion

The published works on ST277 *P. aeruginosa* point to a great similarity between these strains, but these studies are always restricted to the Brazilian strains, almost all mostly SPM-1 producers [2, 8]. Upon our phylogeny results, considering branch length, even if ST277 genomes are closely related we can see a great difference among the strains isolated from China, Mexico and two from EUA when compared to one another. Considering the distribution of resistance genes, PAGIs and type I-C CRISPR-Cas system, these same four genomes were the ones that carry very few of these features. The strains circulating in Brazil have acquired more genetic elements that contribute to their resistance, pathogenicity and success.

The hypothesis that ST277 carries an “intrinsic resistome” has been speculated [34]. However, our findings suggest that the proposed “chromosomal pack of resistance genes” is a characteristic of the ST277 strains circulating mainly in Brazil. Still it was not possible to affirm if this ST appeared as a high-risk clone in this country or if it has already arrived in Brazil with important mechanisms of resistance. The strain collection explored here is of limited size and suffers from a bias in geographical distribution, with high representation of Brazilian strains. Still, this was the most comprehensive genomic analysis of ST277 possible considering the genomes available at the time.

Methods

Selection of ST277 *P. aeruginosa* isolates

Seven clinical *P. aeruginosa* ST277 isolates deposited in the Culture Collection of Hospital-Acquired Bacteria (CCBH) located at the Laboratório de Pesquisa em Infecção Hospitalar Hospital/Fiocruz (LAPIH-IOC) (WDCM947; CGEN022/2010) were sequenced on an Illumina MiSeq instrument (Illumina Inc., San Diego, CA, United States) at the Genomic Lab located in the Departamento de Bioquímica, UERJ (Rio de Janeiro, Brazil). The reads were *de novo* assembled using A5 assembly pipeline [35]. Four of these samples are part of a previous study (CCBH276, CCBH3462, CCBH4851 and CCBH5939) [17]. Posteriorly, CCBH4851 was fully assembled (to be published) (CP021380.1).

Until April 2018, there were 2991 *P. aeruginosa* assembled genomes deposited in NCBI (downloaded on April 2018). To identify the genomes belonging to ST277, we picked up the exact matches resulting from the BLAST (blastn) [36] with the seven alleles for ST277 (retrieved from the MLST databases, <https://pubmlst.org/paeruginosa/>). By this analysis, 36 genomes were assigned to ST277 (1,2%) and included in this study.

ST277 Phylogeny and Pangenome Analysis

For not-completely closed *P. aeruginosa* ST277 genomes (32/43, 74%), the contigs were reordered using the “reorder contigs” option in Mauve, under the default parameters [37]. The strain *P. aeruginosa* CCBH4851 (CP021380.1), deposited in CCBH-IOC, was used as the reference genome [38].

Phylogenetic analysis of the 43 ST277 genomes and the *P. aeruginosa* PAO1 (NC_002516.2) was performed using kSNP3.0 software [39]. The program Kchooser, which is part of the kSNP package, was used to identify the optimal kmer length (23). Dendroscope was used for visualizing and rooting the parsimony tree by using *P. aeruginosa* PAO1 [40].

The GView Server was used to obtain the pangenome of the *P. aeruginosa* PAO1 and the 43 ST277 isolates with a minimum identity of 90% and an $e < 10^{-5}$ [41]. The strain CCBH4851 was used as seed genome. Then, genome comparisons were performed with the BLAST Ring Image Generator (BRIG, 0.95) using the pangenome as a reference [42]. The ST277 PAGIs described in previous works were labeled in this comparison using the coordinates obtained by BLAST (blastn) [36] against the pangenome [8, 17].

Screening of Resistance and Virulence genes in *P. aeruginosa* ST277 Genomes

The prediction of resistome was made using the Comprehensive Antibiotic Resistance database (CARD) [16]. *ln163* was verified by BLASTn [36] searches using GenBank accession AY660529.1. Besides that, BLAST (blastn) alignment results using PAO1 genes as query were analyzed to identify mutations in some genes related to resistance (not included in CARD analyzes) and virulence. Genes were obtained from *Pseudomonas* genome database (<http://www.pseudomonas.com>) [*oprD* (PA0958), *mexT* (PA2492), *mexZ* (PA2020) *lasR* (PA1430), *rhIR* (PA3477), and *algB* (PA5483)]. Moreover, BLAST (blastn) was applied

to check the presence of the virulence genes *exoS* (AY029246.1), *exoU* (KX641457.1), *exoT* (PA0044, PA01), and *exoY* (PA2191, PA01).

CRISPR-Cas and Anti-CRISPR Annotation in *P. aeruginosa* ST277 Genomes

From reordered contigs or fully genomes, CRISPR arrays and their associated proteins (Cas) were predicted using CRISPRCasFinder [43]. The.json file output for each genome was downloaded and used to extract the CRISPR spacers applying in-house Perl scripts. These scripts considered the orientation of the direct repeat and CRISPR to determine the correct orientation of the corresponding spacer sequences. All the CRISPR arrays with more than two spacers had their spacers clustered together using CD-HIT [44] at a 90% identity threshold to obtain a non-redundant spacer set.

Putative anti-CRISPR (*acr*) genes were identified based on BLAST (blastn) [36] matches to nucleotide sequences of anti-CRISPR-associated protein 1 (Aca1, accession YP_007392343). The proteins encoded by genes immediately upstream of putative Aca1 homologue were selected [31]. Eighteen curated *acr* genes were retrieved from previous studies and aligned to the putative *acr* identified here using MUSCLE software [45] [anti-I-F (n = 12), anti-I-E (n = 4), anti-I-C (n = 1) and anti-VA (n = 1)] [31, 33, 46, 47].

Target Identification for Spacer from Type I-C CRISPR-Cas System Located at *P. aeruginosa* ST277 Genomes

When using the non-redundant spacer set as BLAT query (version: 36, parameters: blat -tileSize = 8 - minScore = 28 [reference] [query] [results]) (Kent 2002), following Belkum et al. 2015 [9], we identified the spacer matches against 3 categories of sequence databases: 1) phage genomes from NCBI (n = 4634 phage files, source: <ftp://ftp.ncbi.nlm.nih.gov/genomes/genbank/viral/>) (downloaded on April 2018), 2) Refseq plasmids from NCBI (n = 13428 sequences, source: <ftp://ftp.ncbi.nlm.nih.gov/refseq/release/plasmid/>) (downloaded on April 2018), and 3) the ST277 genomes studied here.

Characterization of Other *P. aeruginosa* Genomes with Type I-C CRISPR-Cas System

To identify the genomes from non-ST277, that carry the operon to Cas system type I-C, the 2991 NCBI *P. aeruginosa* genomes were submitted to BLAST (blastn) [36] against CCBH4851 Cas operon (2,414,915 - 2,422,525 bp). The 57 positive genomes were uploaded at MLST 2.0 [48] available at the Center for Genomic Epidemiology website for in silico MLST (<https://cge.cbs.dtu.dk/services/>).

After MLST, CRISPR arrays and their associated proteins (Cas) were predicted for these genomes using CRISPRCasFinder [43]. The spacers were obtained as described above. We also checked if type I-C CRISPR-Cas systems was inserted in PAGI-34 as described previously [8], using PA3448 (2,091,259–2,197,481 bp) as a reference.

Additional Files

Additional file 1: General information about *P. aeruginosa* NCBI genomes. (XLSX)

Additional file 2: Spacers from *P. aeruginosa* CRISPR-Cas system type I-C. (XLSX)

Additional file 3: BLAT output from *P. aeruginosa* ST277 spacers against plasmids and phages. (XLSX)

Additional file 4: Acr putative sequences. (XLSX)

Additional file 5: Acr nucleotide sequences from *P. aeruginosa* ST277 genomes. (PDF)

Additional file 6: Acr nucleotide sequences from plasmids. (PDF)

Additional file 7: Acr nucleotide sequences from phages. (PDF)

Additional file 8: *P. aeruginosa* ST277 pangenome. (TIFF)

Additional file 9: BLAST output from PAGI-34 against different *P. aeruginosa* clones carrying type I-C CRISPR-Cas subtype. (TIFF)

Abbreviations

CCBH: Culture Collection of Hospital-Acquired Bacteria; CRISPR: Clustered Regularly Interspaced Short Palindromic Repeat; ICE: Integrative and Conjugative Element; MLST: Multilocus Sequence Typing; PAGI: *Pseudomonas aeruginosa* genomic island; ST: Sequence type; SNP: Single Nucleotide Polymorphism.

Declarations

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Availability of data and materials

The genomes are available at GenBank - NCBI, and accession number are in Figure 1. Most other relevant data are contained in Additional files. Other datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

APDC conceived the research; MCS and RMA participated in method design and data handling; MCS performed the data analyses; APDC, MCS and ICOS participated in the selection of sequenced strains; APDC, MCS, CMR wrote parts and edited the complete manuscript.

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Ethics approval and consent to participate

Not applicable. We have no human or animal data involved.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

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Table

Table 1

Mechanism	Resistance	Percentage of positive strains
bla _{OXA-50}	Beta-lactam	100%
PDC-5	Beta-lactam	100%
aph(3')-IIb	Aminoglycoside	100%
catB7	Chloramphenicol	100%
MexEF-OprN (nfxC mutant)	Fluoroquinolones	100%
	Chloramphenicol	
bcr1	Bicyclomycin	100%
fosA	Fosfomycin	100%
GyrA mutation	Fluoroquinolones	93%
aadA7	Aminoglycoside	90%
aacA4	Aminoglycoside	90%
bla _{OXA-56}	Beta-lactam	90%
MexXY-OprM (mexZ deletion)	Macrolides	88%
	Tetracyclines	
	Beta-lactam	
OprD frameshift	Carbapenem	88%
bla _{SPM-1}	Carbapenem	70%
rmtD	Aminoglycoside	65%
crpP	Ciprofloxacin	42%

Figures

Figure 2

Spacer content among *P. aeruginosa* carrying type I-C CRISPR-Cas system. The numbered spacers belong to the ST277 standard spacers content. Colored and numbered spacers are those shared between four different clones (ST277, 235, 17 and 853).

Figure 3

Number of ST277 spacers' matches against public sequences of plasmid and phage. X: Spacers; Y: Number of matches.

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