Title: Evolution of gentamicin and arsenite resistance acquisition in *Ralstonia pickettii* water isolates

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Abstract

Ralstonia pickettii are ubiquitous in water environments. Members of this species are frequently, but not always, resistant to both gentamicin and arsenite. Gentamicin and arsenite co-resistance and the putative molecular mechanisms were investigated. A group of 37 *R. pickettii* strains isolated from drinking water and hospital wastewater were characterized for gentamicin and arsenite resistance phenotypes, the number and size of plasmids, and screened for genetic elements associated with arsenite tolerance, Integrative and Conjugative Elements (ICEs), among other. The genomes of three representative strains were compared.

Most gentamicin resistant (GR) isolates (32/33) were resistant to arsenite, and harbored ICE- and *ars* operon-related genes. These genetic elements were not detected in any of the five arsenite susceptible strains, regardless of the GR (n=1) or gentamicin susceptibility (GS) (n=4) phenotype. The comparison of the genomes of two GR (one resistant and one susceptible to arsenite) and one GS strains suggested that these phenotypes correspond to three phylogroups, distinguished by presence of some genes only in GR isolates, in addition to point mutations in functional genes. The presence of ICEs and *ars* operon-related genes suggest that arsenite resistance might have been acquired by GR lineages.

INTRODUCTION

Members of the species *Ralstonia pickettii*, within the family *Burkholderiaceae* of the class *Betaproteobacteria*, are ubiquitous in aquatic habitats, thriving in wastewater, surface water, and drinking mineral and tap water systems (Becerra-Castro et al., 2015; Falcone-Dias et al., 2012; Ryan et al. 2011; Vaz-Moreira et al., 2017). *R. pickettii* is not considered a primary pathogen and its virulence is considered low, reasons why this species is not screened in routine clinical analyses (Gilligan et al. 2003). However, *R. pickettii* infections have been reported in the literature, indicating that this may be often a misidentified opportunistic pathogen, eventually with a higher incidence than normally assumed. Examples of these situations are meningitis, septic arthritis and osteomyelitis in immunocompromised and cystic fibrosis patients (Coenye et al., 2002; Daxboeck et al., 2005; Ryan and Adley, 2013; Ryan et al., 2006; Stelzmueller et al., 2006; Zellweger et al., 2004). These are unusual, sometimes highly invasive, and severe infections (Waugh et al., 2010). The oligotrophic character of these bacteria might explain the association of nosocomial outbreaks with mineral solutions such as supposedly sterile saline solution, disinfectant or other medical solutions, or from environmental sources, specifically, purified water supplies (CDC, 1998; Riley and Weaver, 1975; Ryan and Adley, 2013). However, *R. pickettii* have also been isolated from a variety of clinical specimens, including nutrient rich fluids and mucous such as sputum, blood, wounds, urine, ear and nose swabs, or cerebrospinal fluid (Stelzmueller et al., 2006). *R. pickettii* are described as being resistant to some antibiotics and disinfectants, as aminoglycosides (amikacin and gentamicin), aztreonam, colistin, ceftazidime, piperacillin–tazobactam, imipenem–cilastatin, ciprofloxacin, and sulphamethoxazole-trimethoprim (Birlutiu et al., 2017; Ferro

et al., 2018; Mijnendonckx et al., 2013; Paterson & Gross, 2018; Vaz-Moreira et al., 2016; Zellweger et al., 2004, Stelzmueller et al., 2006) or chlorhexidine (Weber et al., 2007).

Although members of this species isolated from pristine environments such as mineral water, yield multidrug resistance (MDR) phenotypes (Falcone-Dias et al., 2012), the current knowledge about the nature of antibiotic resistance, including if it is acquired or intrinsic is scarce (Ryan & Adley, 2013). In a previous study with five *Ralstonia* spp. isolates, gentamicin resistance (GR) was observed to coincide with increased arsenite tolerance (Ferro et al., 2019). Since a variable aminoglycoside susceptibility phenotype has been reported in *R. pickettii* (Stelzmueller et al., 2006), the association of both phenotypes suggested that they could have been acquired simultaneously. However, the observation that aminoglycosides and arsenite had distinct effects on growth kinetics, cross-resistance, meaning a common resistance mechanism, was discarded by Ferro et al. (2018). Nonetheless, co-resistance due to genetic linkage might explain the co-occurrence of both phenotypes. This hypothesis drove this study that aimed to investigate the phylogenetic and molecular basis of the observed gentamicin and arsenite co-resistance in wastewater and drinking mineral and tap water *R. pickettii* isolates.

MATERIAL AND METHODS

Bacterial strains

Thirty-seven *Ralstonia pickettii* isolates available in the culture collection of the group were selected for this study: 6 from hospital wastewater, 14 from bottled mineral drinking water, and 17 from treated tap water (Table 1) (Becerra-Castro et al., 2015; Falcone-Dias et al.,

2012; Vaz-Moreira et al., 2013). Plate Count Agar (PCA) was used for routine culturing and Luria-Bertani broth supplemented with 15% (v/v) glycerol for long-term preservation (-80 ºC). Strains were identified based on the 16S rRNA gene sequence analysis, after amplification with the primers 27F and 1492R (Lane, 1991), and comparison with the public database EzBioCloud (Yoon et al., 2017).

Antibiotic resistance phenotypes

Resistance phenotypes were determined based on the disk diffusion method, incubated for 24 h at 30 ºC, as recommended by the Clinical Laboratory Standards Institute (CLSI, 2015) for 17 antibiotics or combinations: aminoglycosides (gentamicin GEN, 10 μg; amikacin AK, 30 μg; kanamycin K, 30 μg; neomycin N, 10 μg; netilmicin NET, 30 μg; tobramycin TOB, 10 μg; streptomycin STR, 10 μg), carbapenems (meropenem MER, 10 μg), cephalosporins (cephalothin CP, 30 μg; ceftazidime CEF, 30 μg), penicillins (ticarcillin TIC, 75 μg), polypeptides (colistin sulphate CT, 50 μg), quinolones/fluoroquinolones (ciprofloxacin CIP, 5 μg; nalidixic acid NA, 30 μg), sulfonamides (sulfamethoxazole SUL, 25 μg; sulfamethoxazole/trimethoprim SXT, 23.75/1.25 μg), and tetracyclines (tetracycline TET, 30 μg). The interpretation criteria (R, resistant; S, susceptible) based on inhibition zone diameters were as follows (mm): GEN10: $R \leq 12$, $S \geq 15$; AK30: $R \leq 14$, $S \geq 17$; K30: $R \leq 13$, $S \geq 18$; N10: $R \leq 12$, $S \geq 15$; NET30: $R \leq 12$, $S \geq 15$; TOB10: $R \leq 12$, $S \geq 15$; STR10: $R \leq 11$, $S \geq 15$; MER10: $R \leq 15$, $S \geq 19$; CP30: $R \leq 14$, $S \geq 18$; CEF30: $R \leq 14$, $S \geq 18$; TIC75: $R \leq 15$, $S \geq 24$; CT50: $R \leq 10$, $S \geq 11$; CIP5: $R \leq 15$, $S \geq 21$; NA30: $R \leq 13$, $S \geq 19$; SUL25: $R \leq 12$, $S \geq 17$; SXT25: $R \leq 10$, $S \geq 16$; TET30: $R \leq 11$, $S \geq 15$. In each assay, the reference strain *Pseudomonas aeruginosa* DSM 1117 was used as quality control.

The minimum inhibitory concentrations (MICs) were determined for gentamicin and arsenite, using the Etest MICE (CN 256–0.015 μg/mL, OXOID, United Kingdom) for gentamicin, or the microdilution method (Andrews, 2001) for arsenite. Microdilution assays used bacterial suspensions in Mueller-Hinton broth supplemented with 0.001–2.0 mM of $NaAsO_2$ with absorbance values at 610 nm of 0.08–0.1.

Screening of selected genetic determinants

Genetic determinants related with: 1) antibiotic resistance (beta-lactamase genes $bla_{OXA\text{-}22}$ and $bla_{OXA\text{-}60}$), 2) arsenite resistance (the *ars* operon and two related genes - *arsH* and *acr3*), 3) efflux pumps (the *cmeA* gene related with an RND efflux pump), and 4) mobile genetic elements (ICEs and plasmids) were screened by PCR. These elements were selected based on literature and also on the comparative analysis of the genomes of GR and GS strains.

The beta-lactamase genes $bla_{\text{OXA-22}}$ and $bla_{\text{OXA-60}}$, frequently described for *Ralstonia* spp. (Girlich et al. 2006) and detected in the genomes of strains H2Cu2 and H2Cu5 (Vaz-Moreira et al., 2016), two of the genes of the *ars* operon, *arsH* and *acr3*, associated to arsenite resistance by detoxification (Yang and Rosen, 2016), and the *cmeA* gene belonging to an RND efflux system, were screened in the 37 *R. pickettii* strains using the primers and annealing temperatures listed in supplementary Table S1. The nucleotide sequences of bla_{OXA} and *cmeA* amplicons obtained from representative GR and GS strains were determined. The deduced amino acid sequences of the *cmeA* encoded membrane fusion protein were compared in GR and GS strains, looking for possible mutations. The

prediction of a possible effect of the amino acids substitutions in the protein function and structure was tested using the PredictProtein 2013 (https://www.predictprotein.org/) (Yachdav et al., 2014). Mega7 software was used for sequences alignment and analysis (Kumar et al., 2016). Putative ICEs were detected based on the conventional PCR screening of five genes (*int*, *traG*, *repA*, *trbI* and *cirlm*), using the primers and conditions recommended by Ryan *et al.* (2009) or optimized for this study (Table S1). The number and size of plasmids was determined by Pulsed-Field Gel Electrophoresis (PFGE), as described before (Ferreira et al., 2019).

Comparative genome analysis

The genomes of two hospital wastewater isolates, one GR (strain H2Cu2, resistant to arsenite) and one GS (strain H2Cu5, susceptible to arsenite), and a GR tap water isolate (T9CP10, susceptible to arsenite), were compared aiming the identification of genetic determinants putatively associated with gentamicin and arsenite resistance phenotypes. The genomes of the strains H2Cu2 and H2Cu5 were sequenced by Ion Torrent PGM and the sequences are deposited in DDBJ/ENA/GenBank under the accession numbers MCGA00000000 and MCGB00000000, respectively (Vaz-Moreira et al., 2016). The genome of the strain T9CP10 was sequenced by Illumina Hiseq, in paired-end mode. Good quality reads were assembled *de novo* with SPAdes v3.6 (http://bioinf.spbau.ru/en/spades). The completeness and contamination of the genome was tested with CheckM (Parks et al., 2015). Contigs longer than 300 bp and with coverage higher than 2, were used for annotation through the RAST server (Aziz et al., 2008). The contigs sequences are

deposited in DDBJ/ENA/GenBank under the accession number JACBXL000000000. Additional information of the three genome sequences is provided in the supplementary Table S3.

The genome sequences of strains H2Cu2, H2Cu5 and T9CP10 and of 10 additional *R. pickettii* isolates available at the NCBI database (access date: June 2020) were compared. The identities of the *R. pickettii* genome sequences were calculated based on the Average Nucleotide Identity (ANI) using the EzBioCloud ContEst16S tool (Lee et al., 2017) (Table S2). The phylogenetic comparison was based on eight housekeeping genes (16S rRNA, *rpoB*, *mutS*, *ppsA*, *adk*, *leuS*, *rplB* and *gyrB*) described for the MLST scheme of *Ralstonia solanacearum* (Tayeb et al., 2008; Wicker et al., 2012). The sequences of the genes were recovered from the genomes, aligned and concatenated using the Bioedit sequence alignment editor (Hall, 1999) and the phylogenetic tree was constructed with Mega version 7 (Kumar et al., 2016).

The genomes of the strains H2Cu2, H2Cu5, and T9CP10 were compared based on the gene prediction and annotation obtained from RAST server (http://rast.nmpdr.org/rast.cgi) for the categories “membrane transport”, “stress response”, “virulence, disease and defense”, and “phages, prophages, transposable elements, and plasmids”. These were selected assuming their possible association with resistance phenotypes. The comparison relied on the search of genetic determinants present only in the GR strains or on the detection of point mutations that distinguished the three genomes (strains H2Cu2, H2Cu5, and T9CP10). The latter compared a set of genes which annotation included the keywords: "RND", "efflux", "secretion", "metal", "antibiotic", or "multidrug". The resultant gene sequences were compared for the three strains based on the nucleotide sequences, using the

185 blastall tool from SAMtools (Li et al., 2009) and the putative associated functions,
186 percentages of similarity and number of possible non-conservative mutations were
187 compared based on deduced amino acids, using the blastp tool
188 (https://blast.ncbi.nlm.nih.gov). Acquired antimicrobial resistance genes were searched
189 using the ResFinder 3.1 search tool (Zankari et al., 2012) with a threshold value of 90%,
190 and plasmids searched through the PlasmidFinder 2.0 tool (Carattoli et al., 2014). Genes
191 related to arsenite resistance were searched in the three genomes annotation and for the
192 prediction of the *ars* operon was used the Operon mapper tool
193 (http://biocomputo.ibt.unam.mx/operon_mapper/) (Taboada et al., 2018). ICEs and IMEs
194 (Integrative and mobilizable elements) were searched in the three genomes using the
195 ICEfinder tool available in the ICEberg 2.0 database webpage (Liu et al., 2019). Insertion
196 sequences (IS) were screened using the ISfinder (https://isfinder.biotoul.fr/) and prophage-
197 related sequences using the PHAge Search Tool (PHAST) (Zhou et al., 2011).

198

199 **RESULTS and DISCUSSION**

200 *Phenotypic characterization and genetic determinants in* Ralstonia pickettii *strains*

201 The *R. pickettii* strains (n=37) isolated from wastewater, tap water, and mineral water
202 (Table 1) were observed to be resistant to the antibiotics streptomycin and colistin sulphate
203 and susceptible to tetracycline, sulfamethoxazole, sulfamethoxazole/trimethoprim,
204 ciprofloxacin, and cephalothin (Table 2). In contrast to what was observed for the
205 aminoglycoside streptomycin, gentamicin resistance was not observed in all isolates. Most
206 of the strains (33 out of the 37), isolated from wastewater, tap water, and mineral water

were resistant to gentamicin (GR) (Table 2), in contrast with four wastewater isolates that were susceptible to this aminoglycoside (GS). GR strains, except the tap water isolate T9CP10, had gentamicin MIC values >256 mg/L and presented increased tolerance to arsenite, with MIC values of 1.4 mM. Strain T9CP10 was the only one combining a gentamicin MIC value >256 mg/L with a low arsenite MIC value of 0.05 mM. An identical arsenite MIC value (0.05 mM) was observed in the GS strains, characterized by gentamicin MIC values ranging 4-8 mg/L.

The comparative 16S rRNA gene sequence analysis originated two groups, one including the four GS strains (100% sequence identity) and the other 32 GR strains (100% sequence identity). Strain T9CP10, GR with a low MIC-arsenite value (0.05 mM), presented 99.8% sequence identity with the GS strains and 99.9% with the other GR strains (Figure S1). This analysis suggested that co-resistance to gentamicin and arsenite might be due to intrinsic mechanisms, related to strains phylogeny.

The genes $bla_{\text{OXA-22}}$ and $bla_{\text{OXA-60}}$ conferring resistance to beta-lactam antibiotics could be amplified in all *R. pickettii* strains, as could be expected according to literature (Girlich et al., 2006, 2004; Nordmann et al., 2000). The respective nucleotide sequences evidenced the existence of allelic variants in GR and GS strains (data not shown), consistent with the gentamicin and arsenite resistance profiles and with the phylogenetic analysis (Figure 1). Jiang et al. (2020) reported two novel allelic variants of the genes $bla_{\text{OXA-22}}$ and $bla_{\text{OXA-60}}$ ($bla_{\text{OXA-899}}$ and $bla_{\text{OXA-898}}$, respectively) in *R. pickettii* strain PSLESD1. Although this strain is closely related to the GR strain H2Cu2, the allelic variants of these two genes are distinct, with nucleotide and amino acid similarities of 97.6% and 98.6% for *bla*OXA-22 and 98.8% and 98.5% for *bla*OXA-60, respectively.

The genes *arsH* and *acr3*, components of the *ars* operon, were detected only in the 32 GR strains that were also resistant to arsenite. These genes were not detected in the GR and arsenite susceptible strain T9CP10 (Table 2). The gene *cmeA* described in *Ralstonia* and other genera, homologous to the gene *amrA* that in *Burkholderia* and *Pseudomonas* spp. has been associated with gentamicin resistance (Podnecky et al., 2015; Poole, 2004), was a possible candidate to explain gentamicin resistance in GR strains. This gene encodes a membrane fusion protein, a component of an efflux pump system of the Resistance-Nodulation-Division (RND), a family of transporters of antibiotics and other chemotherapeutic agents widespread among Gram-negative bacteria (Nikaido and Takatsuka, 2009; Poole, 2004). The *cmeA* gene was detected in all the *R. pickettii* strains, irrespective of the GR or GS phenotype, although with distinct deduced amino acid sequences. In a total of 19 differences in the amino acid sequences observed (Figure 2), 12 were non-conservative and could be putatively associated with gentamicin resistance. Mutations putatively meaningful for gentamicin resistance were the substitution in GR strains, T9CP10 included, in comparison with the GS strains of alanine → glycine (position 9), serine → alanine (positions 134), threonine → proline (position 188), glutamine → lysine (position 227), threonine → alanine (position 253 and 355), glutamine → arginine (position 321), and glycine → serine (position 337) (Figure 2). The observed amino acid substitutions may have implications on the protein structure. According to the PredictProtein tool, alterations in some of the predicted protein binding sites might result from the non-conservative substitutions observed mainly in the region of positions 188-337 (data not shown). A hypothetic substrate range modification due to these mutations would need to be validated, for example, based on protein structure by crystallography.

GR and GS strains differed on the number of plasmids. In general, in GR strains was observed a single plasmid and in GS two plasmids. In GR isolates was observed a single plasmid with 244 kbp or 180 kbp, or of 260 kbp in strain T9CP10. The only exception was strain T6BT1 in which plasmids were not detected. In GS strains were detected two plasmids, one with 75 kbp and another with 244 kbp or 260 kbp (Table 2). The number and size of plasmids did not suggest any relationship with the observed resistance phenotypes or with horizontal gene transfer. The screening of ICEs was another approach to explore possible gene acquisition events in these strains. Indeed, the detection of ICEs-related genetic determinants in all arsenite resistant strains (MIC value of 1.4 mM) (Table 2) raised the hypothesis that arsenite resistance acquisition might have occurred by horizontal gene transfer, as has been described for different prokaryotic groups (e.g. *E. coli*, *Yersinia* spp., *Acidiphilium multivorans*, *Serratia marcescens*, *Halobacterium* sp., *Sinorhizobium* sp., *Bacillus subtilis*, *Acidithiobacillus caldus*, *Leptospirillum ferriphilum*) (Fekih et al., 2018).

In summary, the 37 *R. pickettii* were divided in two groups, one of GR strains with a single plasmid, ICEs-related genes and arsenite resistance (n=32), another of GS strains with two plasmids and without ICEs-related genes and susceptible to arsenite (Table 2). In addition, strain T9CP10 was the only GR strain, susceptible to arsenite, and lacking ICEs-related genes. This observation motivated the comparative analyses of the genome of three strains (Table 2).

Ralstonia pickettii *phylogeny*

The co-resistance to gentamicin and arsenite might be related to the strain's phylogeny as it was suggested by the analyses based on the 16S rRNA gene (Figure S1) and on the concatenation of eight housekeeping genes (Figure 1). Also, the Average Nucleotide Identity (ANI) was higher between strain T9CP10 and each of the strains H2Cu2 (GR) and H2Cu5 (GS) than between these two strains (Table S2). Although no information is available regarding gentamicin resistance phenotypes for most of the strains for which genomes are available, strain PSLESD1, the closest to the GR strain H2Cu2, was described as resistant to gentamicin and amikacin (Jiang et al., 2020). This supports the hypothesis that gentamicin resistance might be associated with strain phylogeny.

Genetic comparison of strains H2Cu2, H2Cu5 and T9CP10

The genomes of strains H2Cu2 (gentamicin and arsenite resistant) and H2Cu5 (gentamicin and arsenite susceptible) and T9CP10 (gentamicin and arsenite susceptible) were further analyzed (Table S3). The Resfinder search revealed the presence of the two beta-lactamases $bla_{\text{OXA-22}}$ and $bla_{\text{OXA-60}}$, while no gentamicin or aminoglycosides resistance genes were identified by this tool. The comparison based on the screening of functional categories sought to identify genes present only in the GR strains or on the detection of point mutations differing in GR and GS strains.

Genes detected in the genomes of the two GR strains (H2Cu2 and T9CP10) but not in the GS strain (H2Cu5), hypothetically associated with gentamicin resistance included genes associated with transporter systems (Table 3). The Tripartite ATP-independent periplasmic (TRAP) transporters use ion electrochemical gradients to move substrates (e.g. C4-dicarboxylates, acidic amino acids, and maybe sugars) in a symporter mechanism (Rosa et

297 al., 2018). Their relationship with antibiotic resistance has never been established, although
298 the knowledge about this class of transporters is sparse (Rosa et al., 2018; von Rozycki et
299 al., 2005). Other transporter related genes were annotated as ABC and Ton/Tol transporters,
300 observed in the GR strain T9CP10 and H2Cu2, respectively. The ABC-type efflux systems
301 are related to the export of drugs (e.g. aminoglycosides, macrolides, lincosamides and/or
302 streptogramins, polymyxins), complex carbohydrates, heme, proteins, and heavy metals
303 (Butaye et al., 2003; von Rozycki et al., 2005). The TonB system was previously described
304 in *Cupriavidus metallidurans* (formerly *Ralstonia metallidurans*) associated with solutes
305 uptake (von Rozycki et al., 2005), but also related to drugs extrusion in *Pseudomonas* spp.
306 (Godoy et al., 2001; Poole, 2001).
307 Genes putatively encoding type IV, VI, and VII secretion systems were only detected in the
308 gentamicin and arsenite resistant strain H2Cu2 (Table 3). This finding is interesting since
309 type IV secretion systems (T4SS) form a membrane-spanning secretion channel and often
310 an extracellular component such as a pilus, that allows the transport of DNA between cells
311 (Liu et al., 2019; Wozniak and Waldor, 2010). T4SS have been associated with antibiotic
312 resistance acquisition in *Betaproteobacteria*, which may happen by the mediation of DNA
313 transfer or through DNA-uptake and -release systems (Cascales and Christie, 2013). Also,
314 T4SS are frequently responsible for the ICEs DNA transfer in Gram-negative bacteria. The
315 type VI secretion systems (T6SS), important for pathogenesis and bacterial competition, are
316 broadly distributed in *Proteobacteria* with roles associated with translocation of toxic
317 effector proteins into prokaryotic cells (Costa et al., 2015). Zhang et al. (2011)
318 demonstrated the association of T6SS with increased resistance of *Pseudomonas*
319 *aeruginosa* biofilms to some antibiotics. However, the major function described for T6SS
320 is the injection of toxins into the neighboring cells to eliminate competition, working as a

weapon of social control in complex microbial communities, and also as a way to have access to the DNA released by killed bacteria and integrate valuable genes and rapidly evolve, leading for example to antibiotic resistance or virulence acquisition (Borgeaud et al., 2015). Type VII secretion systems (T7SS) are specialized secretion systems that are required for the virulence of mycobacteria and that have been detected in several other Gram-positive bacteria (Abdallah et al., 2007; Costa et al., 2015). Curiously, these secretion systems are not described in *Proteobacteria*. In the GR strain H2Cu2 the T7SS was incomplete, with only two genes related to the formation of a pilus and chaperone protein being annotated.

Transduction is considered one of the most relevant processes of antibiotic resistance acquisition by horizontal gene transfer (Salmond and Fineran, 2015). Although the three strains presented genes belonging to the functional category “phages, prophages, transposable elements, and plasmids”, they were distinct. The search for prophages sequences using the PHAST tool identified in the GR strain T9CP10 a region with high degree of identity with the integrative *Ralstonia* phiRSA1 phage (acc. number NC_009382.1) (Fujiwara et al., 2008). The same phage was detected in the GR strain H2Cu2, but incomplete. Other incomplete prophages were identified in the GR strain T9CP10 (*Ralstonia* phage RSK1, acc. number NC_022915; *Rhodoferax* phage P26218, acc. number NC_029061) and in the GS strain H2Cu5 (*Gordonia* phage Nymphadora, acc. number NC_031061; *Loktanella* phage pCB2051-A, acc. number NC_020853).

Another important feature detected in the gentamicin and arsenite resistant strain H2Cu2 was the *ars* operon (Figure 3), not detected in the arsenite susceptible strains H2Cu5 or T9CP10. Other genes observed only in strain H2Cu2 were related to the uptake of selenate and selenite, to mechanisms of regulation of the osmotic stress, or to lysozyme inhibitors.

Also, the GR strain T9CP10 presented some genes, such as *chrC* and *chrF*, related to oxidative stress response and resistance to mercury and chromium compounds that were not detected in the other strains (Table 3). The distinctive profile of genes may be related to specific functions observed in the three isolates, hinting distinct evolution paths, although a deeper genome analysis would be needed to confirm this hypothesis.

In order to get additional insight of this comparative genome analysis, point mutations in genes belonging to the functional categories "membrane transport", "stress response", "virulence, disease and defense", and "phages, prophages, transposable elements, and plasmids" were searched for. It was observed that 68 out of 72 deduced amino acid protein sequences that were common to GR and GS strains had point mutations (Table S4), while only four had 100% amino acid sequence identity in the three genomes. The only category for which no common proteins were found was "phages, prophages, transposable elements, and plasmids". For the others (n=68) were considered those with higher percentage of dissimilarity, mainly due to non-conservative mutations, hypothetically those that might be associated with an altered phenotype. Using this rationale, were identified 18 proteins, with 4% or more of non-conservative mutations. Most of these were related to metals tolerance (copper, zinc, chromium and mercury) and secretion systems (Table S4). In particular, for proteins associated with mercury resistance a high similarity between strains GR H2Cu2 and GS H2Cu5, and dissimilarity with strain T9CP10 was observed (Table S4). The RND efflux pump genes *cmeA, cmeB,* and *cmeC* yielded dissimilarity values between the three strains below or close to 4% (Table S4). However, not only the number of mutations but their location in the protein structure will determine the effect they may have, as was observed for the CmeA protein structure prediction obtained with the PredictProtein. For that reason, this group of proteins deserves further investigation to unveil the possible effect

of the observed mutations in the strain's tolerance to metals or antimicrobials. Also, the expression of these genes, regulated by the CmeR transcriptional repressor (Grinnage-Pulley and Zhang, 2015; Lin et al., 2005) may explain distinct resistance phenotypes. In this aspect, the transcriptome analysis under different conditions, including the presence and absence of gentamicin, may also bring important inputs to the understanding of the resistance mechanism.

A functional *ars* operon, with approximately 7 kbp, with the structure described in Figure 3 was assembled from the genome of strain H2Cu2. The structure of this *ars* operon includes seven genes previously (Fekih et al., 2018) described as members of arsenite resistance operons: *arsR* (a transcriptional regulator), two *arsC* (arsenate reductases, able to transform arsenate to arsenite), *arsD* (responsible for bind arsenite and transfer it to the ArsA ATPase prior to the oxyanion extrusion), *arsA* (ATPase responsible for the arsenite efflux), *acr3* (arsenite efflux pump), and *arsH* (organoarsenical oxidase enzyme). In the structure of the operon was also identified a lactoylglutathione lyase (EC 4.4.1.5) and a hypothetical protein (FIG00460211) (Figure 3).

The GS strain H2Cu5 and the GR strain T9CP10 harboured three genes that could be related to arsenite resistance. These genes were the transcriptional regulator *arsR*, the arsenical pump membrane protein (*arsB*), and the arsenate reductase (*arsC*). However, to be functional, these three genes need to be organized in an operon arsRBC, which was not the case in these two isolates. To be able to confer resistance to arsenite, the ArsB pumps need to be energized by ATP hydrolysis, which is catalysed by *arsA*, or by motive force in arsRBC operons (Fekih et al., 2018). It is curious to note the presence of the genes *arsR*, *arsB* and *arsC* in the genomes of the arsenite susceptible strains, since the arsRB and the

later arsRBC operons are among the most primitive mechanisms of resistance to arsenite (Fekih et al., 2018).

The genetic environment of the *ars* operon in the genome of strain H2Cu2 suggested the vicinity of mobile genetic elements. The association between arsenite resistance and the presence of the ICEs genetic determinants (Table 2) was investigated using the tool ICEfinder to screen ICEs related genes in the contig where *ars* operon was detected. This search revealed the presence of a putative ICE, about 12 kbp upstream to the *ars* operon. This ICE, identified based on the ICEfinder, had 75.2% sequence identity (for query coverage of 85%) with an ICE described in *Achromobacter xylosoxidans* strain A8 (ICEfinder score: 41). ICEfinder revealed a second ICE in the genome of strain H2Cu2, which was 89.1% similar (although with an extremely low query coverage of 12%) to that reported in the *R. pickettii* 12J (ICEfinder score: 39) by Ryan *et al*. (2009) and that was used in this study to design the ICE screening PCR primers (Table S1). The analysis of the genome of strain H2Cu2 did not reveal any cargo genes possibly related to gentamicin or arsenite resistance within the ICE structure. However, in both predicted ICEs were identified the recombination sites (*attL* and *attR*) demonstrating that these elements still have the capacity to be mobilized (Landy and Ross, 1977). Although in the genome of strains H2Cu5 and T9CP10 it was possible to detect elements that typically form an ICE, such as the relaxase, and some T4SS elements, some others were missing.

From the results obtained it is possible to conclude that:

- Gentamicin resistance may be intrinsic in *R. pickettii* and related to specific phylogenetic lineages.

- Due to phenotype or phylogeny, gentamicin resistant *R. pickettii* strains may have facilitated access to acquire arsenite resistance genes. Indeed, elements such as T6SS, T4SS and phage related genes, were observed only in the gentamicin resistant strains.
- Moreover, the *ars* operon and ICEs related genes were only observed in gentamicin resistant strains, which yield also (putatively acquired) arsenite resistance.

The analysis of the phylogeny, phenotypes and genotypes of *R. pickettii* from different aquatic environments supported the conclusion that the hypothesized association between gentamicin resistance and increased arsenite tolerance is a consequence of strain phylogeny, which in turn may have different histories of resistance acquisition. This may result from co-evolution or co-selection of the two mechanisms of resistance, excluding the possibility of a common mechanism of resistance for the two antimicrobials (cross-resistance). The increased tolerance to arsenite seems to be associated to the presence of ICEs.

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667 Table 1. *Ralstonia pickettii* isolates tested in this study

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Source	Number of strains	Isolation or evolution conditions	Reference
Mineral water	14	Pseudomonas isolation agar with 32 mg/L amoxicillin	(Falcone-Dias et al., 2012)
Tap water	17	Tergitol 7-agar	(Vaz-Moreira et al., 2013)
Hospital wastewater	6	Culture enrichment in modified Luria-Bertani broth with Cu^{2+} (2.5 mM)	(Becerra-Castro et al., 2015)

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672 Table 2. Phenotypic and genotypic characterisation of the *Ralstonia pickettii* strains for antibiotics and arsenite tolerance, and presence
673 of the genetic elements ICEs, plasmids and the arsenite resistance related genes *acr3* and *arsH*. In bold are indicated the strains that
674 were used for the genomic and phylogenetic comparisons.

Source	Strains	Antibiotic resistance phenotypes						MICs		ICEs	Plasmid size (kbp)	Arsenite related genes	
		STR	AMG	TIC	CEF	MER	NA	GEN (mg/L)	As^{3+} (mM)			*acr3*	*arsH*
Mineral water	L1R01, L1R03	R	R	R	S	I	I	>256	1.4	+	244	+	+
	L1R02, L1R04, L1PA4, L1PA5	R	R	R	S	S	S	>256	1.4	+	244	+	+
	L1RA2	R	R	R	S	I	S	>256	1.4	+	244	+	+
	L1RA3	R	R	R	I	R	I	>256	1.4	+	244	+	+
	L1P02, L1PA1	R	R	R	S	R	I	>256	1.4	+	244	+	+
	L1P05	R	R	R	S	S	I	>256	1.4	+	244	+	+
	L3P01	R	R	R	S	R	S	>256	1.4	+	180	+	+
	L2P04, L3P03	R	R	I	S	S	S	>256	1.4	+	180	+	+
Tap	T1BT2, T1BT11, T3BT8	R	R	R	S	I	I	>256	1.4	+	180	+	+
	T1BT6	R	R	R	S	I	S	>256	1.4	+	180	+	+
	T6BT1	R	R	R	S	I	S	>256	1.4	+	-	+	+
	T3BT1, T6BR7, T7BT3, T7BT10, T7BT16, T7BP2	R	R	R	S	R	I	>256	1.4	+	180	+	+
	T3BT7, T3BT13, T3BP5, T7BT7, T7BP20	R	R	R	I	R	S	>256	1.4	+	180	+	+
	T9CP10	R	R	S	S	S	S	>256	0.05	-	260	-	-

Wastewater	**H2Cu2**	R	R	R	S	R	I	>256	1.4	+	244	+	+
	H2Cu4	R	R	R	S	R	R	>256	1.4	+	244	+	+
	H2Cu3	R	S	R	S	S	I	4	0.05	-	75, 260	-	-
	H2Cu5	R	S	R	I	I	I	8	0.05	-	75, 244	-	-
	H2Cu7	R	S	R	S	R	I	6	0.05	-	75, 260	-	-
	H2Cu8	R	S	R	S	R	S	6	0.05	-	75, 260	-	-

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676 STR, streptomycin; AMG, other Aminoglycosides (GEN, gentamicin; AMK, amikacin; KAN, kanamycin; NEO, neomycin; NET, netilmycin;
677 TOB, tobramycin); TIC, ticarcillin; CEF, ceftazidime; MER, meropenem; NA, nalidixic acid. As^{3+}, arsenite. ICEs, integrative and conjugative
678 elements; +, detected; -, non-detected

679 All strains were susceptible to: TET, tetracycline; SUL, sulfamethoxazole; SXT, sulfamethoxazole/trimethoprim; CIP, ciprofloxacin; CP,
680 cephalothin. All strains were resistant to STR, streptomycin and CT, colistin sulphate.

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684 Table 3: Genes only present in the genomes of the gentamicin resistant strains H2Cu2 and T9CP10.

Category	**Subcategory**	**Subsystem (role or proteins)**	**Strain**
Membrane Transport	Protein and nucleoprotein secretion system, Type IV	Conjugative transfer (TrbC, TrbL)	H2Cu2
Membrane Transport	Protein secretion system, Type VI	Type VI secretion systems (IcmF-related protein, ImpG/VasA, VasD, ImpA, ImpB, ImpC, ImpD, ImpF, ImpH/VasB, ImpJ/VasE)	H2Cu2
Membrane Transport	Protein secretion system, Type VII (Chaperone/Usher pathway, CU)	Type 1 pili (mannose-sensitive fimbriae, gamma-fimbriae FimA); Sigma-Fimbriae chaperone, tip adhesin and usher protein	H2Cu2
Membrane Transport	TRAP transporters	TRAP Transporter collection (TRAP-type C4-dicarboxylate transport system, large permease and periplasmic component)	H2Cu2 and T9CP10
Membrane Transport	ABC transporters	ABC transporter alkylphosphonate (Phosphonate ABC transporter ATP-binding and permease proteins)	T9CP10
Membrane Transport	no subcategory	Ton and Tol transport systems (TPR domain protein, putative component of TonB system and TonB-dependent receptor)	H2Cu2
Stress Response	Detoxification	Uptake of selenate and selenite (various polyols ABC transporter: ATP-binding component; periplasmic substrate-binding protein; permease component 1 and 2)	H2Cu2
Stress Response	Osmotic stress	Choline and Betaine Uptake and Betaine Biosynthesis (Choline-sulfatase)	H2Cu2
Stress Response	Oxidative stress	Oxidative stress (Superoxide dismutase [Mn])	T9CP10
Virulence, Disease and Defense	Resistance to antibiotics and toxic compounds	Arsenic resistance (ArsH, ArsA, ArsD, ACR3)	H2Cu2
Virulence, Disease and Defense	Resistance to antibiotics and toxic compounds	Resistance to chromium compounds (Superoxide dismutase ChrC and SodM-like protein ChrF)	T9CP10
Virulence, Disease and Defense	Resistance to antibiotics and toxic compounds	Mercury resistance (Mercuric transport protein, MerC)	T9CP10

Virulence, Disease and Defense	Resistance to antibiotics and toxic compounds	Lysozyme inhibitors (Inhibitor of vertebrate lysozyme precursor)	H2Cu2
Phages, Prophages, Transposable elements, Plasmids	Phages, Prophages	Phage capsid proteins (scaffolding protein, phage head completion-stabilization protein, major capsid protein)	H2Cu2 and T9CP10
Phages, Prophages, Transposable elements, Plasmids	Phages, Prophages	Phage lysis modules (lysozyme)	T9CP10
Phages, Prophages, Transposable elements, Plasmids	Phages, Prophages	Phage packaging machinery (Phage portal protein, Phage terminase (ATPase, endonuclease and large subunit), Phage-related capsid packaging protein	T9CP10
Phages, Prophages, Transposable elements, Plasmids	Phages, Prophages	Phage replication	T9CP10
Phages, Prophages, Transposable elements, Plasmids	Phages, Prophages	Phage tail fiber proteins	H2Cu2 and T9CP10
Phages, Prophages, Transposable elements, Plasmids	Phages, Prophages	Phage tail proteins (major tube protein, completion protein, length tape-measure protein)	H2Cu2 and T9CP10

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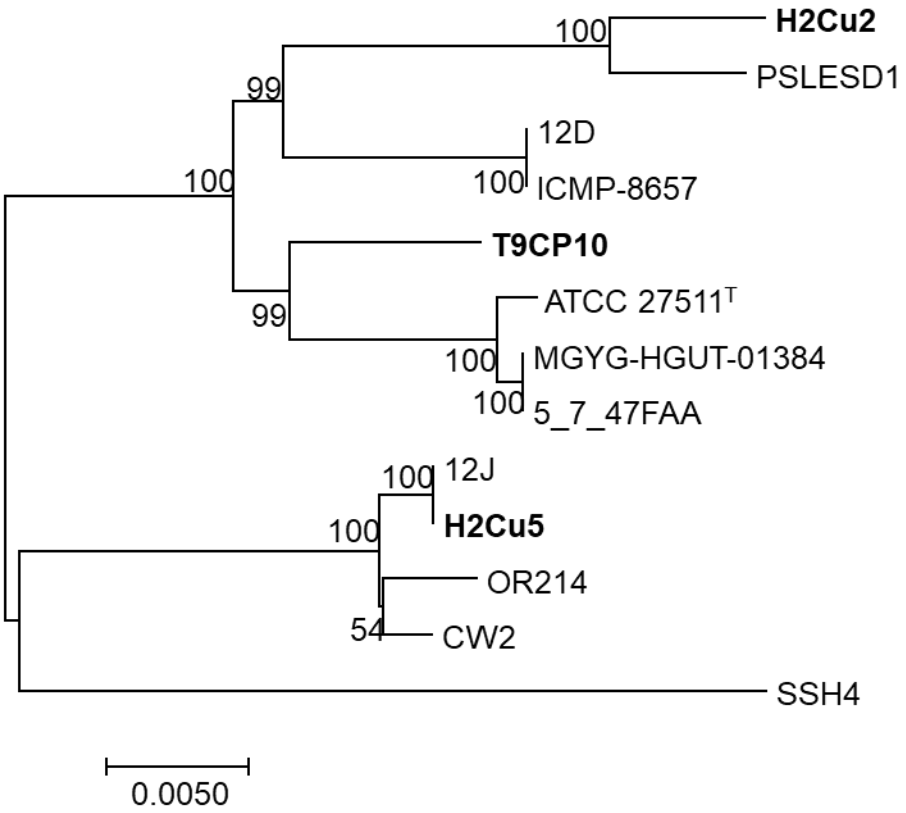

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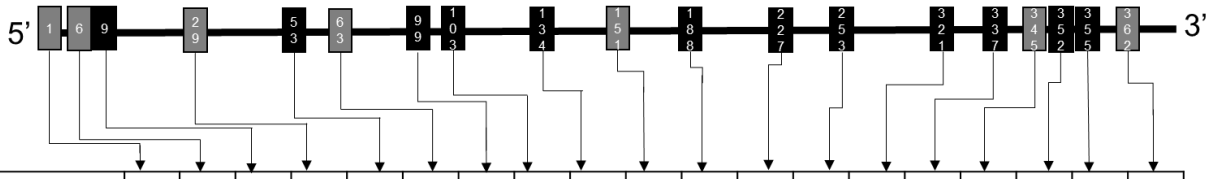
689 Figure 1. Relationship of the three representative *Ralstonia pickettii* strains of this study
690 (in bold) and the ten *Ralstonia pickettii* strains for which genomes are available at the
691 public NCBI GenBank database, based on the analysis of 17200 bp of the concatenated
692 genes 16S rRNA, *rpoB*, *mutS*, *ppsA*, *adk*, *leuS*, *rplB* and *gyrB*.



a.a. position	1	6	9	29	53	63	99	103	134	151	188	227	253	321	337	345	352	355	362
GR (n= 8)	V	V	G	V	E	L	A	S	A	D	P	K	A	R	S	L		A	V
GS (n= 3)	A	V	A	A	Q	L	A	A	S	E	T	Q	T	Q	G	V		T	I
T9CP10 (n= 1)	V	A	G	V	Q	F	T	A	A	D	P	K	A	R	S	L		A	V

GR, gentamicin and arsenite resistant; GS, gentamicin and arsenite susceptible; T9CP10, gentamicin resistant and arsenite susceptible.

Figure 2. Schematic representation of the allelic variants of the RND efflux pump related gene *cmeA*, with indication of the positions where different amino acids are observed. In grey are indicated the conservative differences and in black the non-conservative.

Figure 3. *Ralstonia pickettii* H2Cu2 *ars* operon prediction. For the strains H2Cu5 and T9CP10 were only identified the genes *arsR*, *arsC* and *arsB*, not organised in an operon.

Genes: *arsR* (transcriptional regulator), gloA (Lactoylglutathione lyase (EC 4.4.1.5), *arsC* (arsenate reductase), *arsD* (responsible for bind arsenite and transfer it to the ArsA ATPase prior to the oxyanion extrusion), *arsA* (ATPase responsible for the arsenite efflux), *acr3* (arsenite efflux pump), hyp. prot. (hypothetical protein (FIG00460211), and *arsH* (organoarsenical oxidase enzyme).

Supplementary materials:

Table S1. List of primers used in this study.

Table S2. Average Nucleotide Identity (ANI) values for the *Ralstonia pickettii* genomes available at the NCBI GenBank database.

Table S3: Characteristics of the three genomes compared in this study.

Table S4: Comparison of selected functional categories for the strains H2Cu2 (gentamicin and arsenite resistant), H2Cu5 (gentamicin and arsenite susceptible) and T9CP10 (gentamicin resistant and arsenite susceptible). Comparisons: A) H2Cu2 vs T9CP10; B) H2Cu2 vs H2Cu5; C) T9CP10 vs H2Cu5.

Figure S1. Relationship of *Ralstonia pickettii* strains used in this study and the ten *Ralstonia pickettii* strains for which genomes are available at the public NCBI database (in italics), based on the analysis of 1383 bp of the 16S rRNA gene. The dendrogram was inferred using the Neighbour-Joining method and the evolutionary distances computed using the Jukes-Cantor method in MEGA7 (Kumar et al., 2016).