Title: Association between gentamicin resistance and stress tolerance in water isolates of *Ralstonia pickettii* and *R. mannitolilytica*

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ABSTRACT

Members of the species *Ralstonia pickettii* and *R. mannitolilytica*, although ubiquitous and lacking major virulence factors, have been associated with nosocomial outbreaks. Tolerance to metals, antibiotics and disinfectants may represent an advantage for their ubiquity and opportunistic pathogenic potential. In this study we compared five strains that differed on the origin (hospital effluent, tap water, mineral water) and in the susceptibility to aminoglycosides, regarding their tolerance to metals and disinfection. The growth kinetics and biofilm formation capacity were tested in four *R. pickettii* strains and one *R. mannitolilytica* at sub-inhibitory concentrations of aminoglycosides or arsenite. The survival to UV radiation, chlorine or hydrogen peroxide was also compared in aminoglycoside resistant and susceptible strains. Aminoglycoside resistant strains presented a higher tolerance to arsenite than the susceptible ones and either aminoglycosides or arsenite were observed to stimulate the biofilm formation. Sub-inhibitory concentrations of the aminoglycoside gentamicin or arsenite significantly decreased the growth rate and yield, but only arsenite caused a significant increase of the lag phase. Hydrogen peroxide presented higher disinfection effectiveness against aminoglycoside susceptible than against resistant strains, an effect that was not observed for UV or chlorine. Although this conclusion needs validation based on a larger number of isolates, including clinical, the results suggest that aminoglycoside resistance may be associated with traits that influence *Ralstonia* spp. fitness in the environment.

KEYWORDS

aminoglycosides resistance; biofilm; disinfectants; kinetics; sub-inhibitory concentrations

INTRODUCTION

Members of the phylum *Proteobacteria*, mainly some families and genera of the classes *Alpha-*, *Beta-* and *Gammaproteobacteria* are amongst the most prevalent bacteria in water habitats (Vaz-Moreira et al. 2014; Vaz-Moreira et al. 2017). Some species of the genus *Ralstonia* within the class *Betaproteobacteria*, in particular the species *Ralstonia pickettii* and *R. mannitolilytica*, are frequently observed in aquatic habitats, specifically in wastewater, potable water, surface water and mineral water (Becerra-Castro et al. 2015; Falcone-Dias et al. 2012; Ryan et al. 2011; Vaz-Moreira et al. 2017). These *Ralstonia* species are comprised of ubiquitous bacteria that have been found in a wide variety of environments, such as in plastic (Poly Vinyl Chloride) pipes forming biofilms structures, aerospace samples, purified water, saline solutions, skin disinfectants, biological samples, caps of blood culture bottles or even in human patients in the respiratory tract or the oral cavity (Adley et al. 2005; Anderson et al. 1990; Boutros et al. 2002; Coenye et al. 2002; Coman et al. 2017; Daxboeck et al. 2005; Koenig and Pierson 1997; Kulakov et al. 2002; Labarca et al. 1999; Maroye et al. 2000; McNeil et al. 1985; Mijnendonckx et al. 2013; Riley and Weaver 1975; Ryan et al. 2006; Stelzmueller et al. 2006; Verschraegen et al. 1985). The ubiquitous character of these bacteria is related to the requirement for minimal nutrient resources and explains the transmission from various sources to humans (Daxboeck et al. 2005). The capacity to grow in moist environments and to form biofilm has also been proposed as a reason for the *Ralstonia* spp. persistence in some environments (Adley et al. 2005). Motility, a characteristic of *Ralstonia* spp., is also sometimes associated with the increased capacity to form biofilm (Guttenplan and Kearns 2013; O'Toole and Kolter 1998). Although the decrease of motility after prolonged preservation

and sub-culture has been reported in *Ralstonia* spp. (Ryan 2009; Vaneechoutte et al. 2001), the association between motility and biofilm formation have been proposed in this genus. In particular, in some *Ralstonia* spp. aerotaxia was observed to regulate the biofilm formation (Yao and Allen 2007).

R. pickettii and *R. mannitolilytica* are not considered primary pathogens and, hence, are not screened in routine monitoring analyses in hospitals (Coenye et al. 2002; Orme et al. 2015; Waugh et al. 2010). Nevertheless, it has been argued that the low frequency of infection episodes attributed to *Ralstonia* spp. may be a consequence of misidentifications of these bacteria, being suggested that members of this group may be more widespread, invasive and severe than previously thought (Coman et al. 2017; Daxboeck et al. 2005; Ryan et al. 2006). A recent study reported an association between the presence of intestinal *Ralstonia pickettii* and an augmented glucose intolerance in obesity (Udayappan et al. 2017). Although members of *R. pickettii* and *R. mannitolilytica* lack major virulence factors, and rarely are reported as causing infection, members of these species have been considered the most pathogenic species of the genus (Vaneechoutte et al. 2004). Indeed, nosocomial outbreaks attributed to *R. pickettii* or *R. mannitolilytica* have been reported regularly over the last 30 years (Coman et al. 2017; De Baere et al. 2001; Fernandez et al. 1996; Khajuria et al. 2014; Labarca et al. 1999; Riley and Weaver 1975; Ryan et al. 2006; Vaneechoutte et al. 2001; Verschraegen et al. 1985).

In part, the ubiquitous character and potential to infect humans may be associated with the capability to stand environmental stresses observed in *R. pickettii* and *R. mannitolilytica*. For instance, the capacity to survive in hospital disinfectants, as chlorhexidine and ethacridine lactate (acrinol) (Ryan et al. 2006) or to participate in bioremediation processes

through the breakdown of xenobiotic compounds was described in *R. pickettii* (Ryan et al. 2007). Also metal tolerance seems to be a relevant property of these bacteria illustrated by the fact that the microbiota enriched from a hospital effluent in copper led to the isolation of monospecies cultures of metal resistant *Ralstonia* spp., in spite of the complex microbial community of such an effluent (Becerra-Castro et al. 2015). The array of antibiotic resistance phenotypes to gentamicin, chloramphenicol, colistin, tobramycin, polymyxin B, and many others observed in members of these species are probably also related with their ubiquity (Daxboeck et al. 2005; Pan et al. 2011; Ryan et al. 2009; Stelzmueller et al. 2006).

Given this background information, the occurrence of *R. pickettii* and *R. mannitolilytica* throughout the urban water cycle (wastewater and tap water) and in pristine water sources (mineral water) (Becerra-Castro et al. 2015; Falcone-Dias et al. 2012; Kulakov et al. 2002; Ryan et al. 2006; Vaz-Moreira et al. 2017), raises interest on their ecology. Able to withstand the drinking water treatment, bacteria of these species can reach humans through the water consumption. This work was based on the hypothesis that traits, such as tolerance to metals and antibiotics, to disinfection or the capacity to produce biofilm in the presence of antibiotics, may differ among *Ralstonia* spp. strains and hence affect their response to stress and environmental fitness. As part of the experimental design, a set of five *Ralstonia* spp. strains, isolated from hospital wastewater, mineral water, and tap water, were tested for their response under the abovementioned stress types.

MATERIALS AND METHODS

Bacterial strains

Five *Ralstonia* spp. isolates were selected for this study, four *Ralstonia pickettii*, two from hospital wastewater, one from mineral water and one from tap water, and one *Ralstonia mannitolilytica* from tap water (Table 1). Cultures were maintained and preserved in nutritive (Luria-Bertani) broth supplemented with 15% (v/v) glycerol.

The identification of the strains was made based on the 16S rRNA gene sequence analysis using the primers 27F and 1492R as previously described (Ferreira da Silva et al. 2007). The sequences were compared with the public database EzBioCloud (Yoon et al. 2017). The five strains were characterized based on selected biochemical tests using the commercial kits API 20E, API 20NE, and API ZYM (bioMérieux) following the manufacturer's instructions. Capsule presence was tested by negative staining (McKinney 1953) in cultures grown in the absence and presence of sub-inhibitory concentrations of gentamicin. These additional characterizations were done in an attempt to find some traits that could be associated with the antibiotic and metals resistance phenotypes.

The 16S rRNA gene sequences of the studied strains were compared with other good quality (>1000 bp) sequences of *R. pickettii* and *R. mannitolilytica* strains of different origins available in the GenBank (http://www.ncbi.nlm.nih.gov/). The nucleotide sequence analysis was performed using the MEGA6 software (Tamura et al. 2013), based on the model of Jukes and Cantor (Jukes and Cantor 1969), and a dendrogram was created using the neighbour-joining method. The iTol software v3.2.4 (Letunic and Bork 2016) was used to represent the isolates source in the dendrogram.

Determination of antibiotic and metal resistance phenotypes

The antibiotic resistance phenotypes were determined by disk diffusion method as recommended by the Clinical Laboratory Standards Institute (CLSI 2015), for 12

147 antibiotics: nalidixic acid (NA, 30 μg); ciprofloxacin (CIP, 5 μg); streptomycin (STR, 10
148 μg); gentamicin (GEN, 10 μg); tetracycline (TET, 30 μg); cephalothin (CP, 30 μg);
149 meropenem (MER, 10 μg); ceftazidime (CEF, 30 μg); ticarcillin (TIC, 75 μg); colistin
150 sulphate (CT, 50 μg); sulfamethoxazole (SUL, 25 μg) and sulfamethoxazole/trimethoprim
151 (SXT, 23.75/1.25 μg). The interpretation criteria (R, resistance; S, susceptible) based on
152 inhibition zone diameters were as follows (mm): NA30: R≤13, S≥19; CIP5: R≤15, S≥21;
153 STR10: R≤11, S≥15; GEN10: R≤12, S≥15; TET30: R≤11, S≥15; CP30: R≤14, S≥18;
154 MER10: R≤15, S≥19; CEF30: R≤14, S≥18; TIC75: R≤15, S≥24; CT50: R≤10, S≥11;
155 SUL25: R≤12, S≥17; SXT25: R≤10, S≥16. In each assay, the reference strain *Pseudomonas*
156 *aeruginosa* DSM 1117 was used for quality control.

157 *Determination of Minimum Inhibitory Concentrations* (MICs)

158 The minimum inhibitory concentrations (MICs) were determined using the Etest or the
159 microdilution method at 30 ºC. The Etest (BioMérieux, France) or MICE (OXOID, United
160 Kingdom) were used for the antibiotics gentamicin (CN 256-0.015 μg/mL, OXOID,
161 MA0116F), streptomycin (SM 0.064-1024 μg/mL, BioMérieux, 526800), ceftazidime (TZ
162 0.016-256 μg/mL, BioMérieux, 412293), meropenem (MEM 32-0.002 μg/mL, OXOID,
163 MA0121F), and sulfamethoxazole (SX 0.064-1024 μg/mL, BioMérieux, 412458). The
164 microdilution method was used for tetracycline and metals (Andrews 2001), using bacterial
165 suspensions of absorbance 0.08-0.1 at 625 nm in Mueller-Hinton broth supplemented with
166 0.1 - 32 mg/L of tetracycline, 0.001 - 2 mmol/L of $NaAsO_2$, 0.01 - 10 mmol/L of
167 $NiCl_2 \cdot 6H_2O$ or 1 – 14 mmol/L of $CuSO_4 \cdot 5H_2O$. For concentrations of $CuSO_4 \cdot 5H_2O$ above
168 5 mmol/L the MICs were tested in Tris-buffered Mueller-Hinton broth. The MICs were

169 determined as the minimum concentration that inhibited visible bacterial growth after 24 h
170 of incubation.

171 Based on preliminary distinctive results between the tested strains, the aminoglycoside
172 gentamicin, and the metal arsenite were selected to assess their effects as stressors and will
173 be from this point forward designated as stressors. Each of the five strains was assayed in
174 stressor-free (SF) culture medium and in the presence of gentamicin or arsenite at
175 concentrations close to the MIC value.

176 *Stressors and growth kinetics*

177 Cultures were assayed in Mueller-Hinton broth or in this culture medium supplemented
178 with adequate concentrations and volume of stressor solution. Therefore, strains H2Cu2,
179 T6BT1 and L1PA1 were assayed in 125 mg/L gentamicin or 1.1 mmol/L As^{3+}; strain
180 H2Cu5 was assayed in 6 mg/L gentamicin or 0.01 mmol/L As^{3+}; and strain T6BT10 was
181 assayed in 0.4 mg/L gentamicin or 0.01 mmol/L As^{3+}. Bacterial suspensions with an initial
182 absorbance of 0.05 at 610 nm (A610) were incubated at 30 ºC with orbital shaking (~70
183 rpm) and were monitored every hour until reached the stationary phase (~24 h). Growth
184 curves and kinetic parameters (growth rate, lag phase, and yield) were determined in
185 triplicate in independent assays. Growth curves were represented as log values of A610 in
186 function of time. The lag phase was the period of time necessary to start the exponential
187 phase. The growth rate (μ) was determined based on the slope of the curve during the
188 exponential growth phase, according to the equation $Ln\ N_t - Ln\ N_0 = \mu(t - t_0)$, where N is
189 the number of cells at time t. The growth yield corresponded to the maximum A610
190 reached.

Stressors and biofilm formation

The capacity of each strain to form biofilm was tested in modified Luria-Bertani broth (mLB) (tryptone 5 g/L, yeast extract 2.5 g/L and sodium chloride 1 g/L) over a range of different stressor concentrations: 0.01, 0.05, 0.5 and 1.1 mmol/L As^{3+}; 25, 75 and 125 mg/L of GEN; and 125, 250, 500 and 750 mg/L of STR, concentrations bellow the MICs for the strains H2Cu2, L1PA1 and T6BT1; and of 0.01 mmol/L of As^{3+}; 0.4 and 6 mg/L of GEN; and 50 mg/L of STR for the strains H2Cu5 and T6BT10. The assays were performed in clear flat bottom 96-well polystyrene microtiter plates (Orange Scientific, Belgium) as described by Simões *et al.* (2007). Briefly, the microtiter wells were filled with 200 µL of bacterial suspension (A610 = 0.1; prepared from overnight cultures in mLB at 30 ºC) in mLB or in mLB supplemented with one of the stressors, incubated for 48 h at 30 °C and measured the absorbance at 620 nm (A620) in a microplate reader (FLUOstar optima, BMG Labtech, Germany). After that, the plates were washed with phosphate buffer and air-dried for 30 min. To assess and compare the biofilm formation, the biomass was fixed with methanol, left to dry, stained with crystal violet, washed again and the dye resuspended with glacial acetic acid prior to measuring the absorbance at 570 nm (A570). A negative control consisting of non-inoculated culture medium and a reference culture (*Pseudomonas aeruginosa* DSM 1117) were included in each assay. Each experiment was performed at least six times for each strain. The quantification of the biofilm formation was performed as described by Rode e*t al.* (Rode et al. 2007), through the calculation of a ratio A_{570}/A_{620}, referring to absorbance at 570 nm (to measure the biofilm formation) and absorbance at 620 nm (to measure the bacterial growth). The absorbance values were corrected by the subtraction of the respective absorbance measured in the negative control (non-inoculated

214 culture medium). With the procedure used, the possible contribution of the growth yield for
215 the capacity to form biofilm was normalized by the use of the ratio A_{570}/A_{620}, referring to
216 absorbance at 570 nm (measure of the biofilm formation) and absorbance at 620 nm
217 (measure of the bacterial growth).

218 *Disinfectants and inactivation*

219 The effectiveness of the germicide UV radiation, chlorine or hydrogen peroxide, was tested
220 in saline solution (0.85% (w/v) NaCl) bacterial suspensions of A610 = 0.1. Suspensions
221 were prepared from 24 h Plate Count Agar (PCA) cultures. Samples collected at the
222 beginning and over the assay were cultivated for enumeration on PCA and incubated at 30
223 ºC for 24-48 h.

224 For UV disinfection was used a germicide UV lamp with a wavelength of 254 nm, under
225 which were exposed PCA plates onto which were spread 100 µL of a bacterial suspension
226 with about 10 to 300 CFU/mL. Exposure times were of periods of 0, 15, 30, 45, 60, 90 and
227 150 seconds.

228 To test the effect of chlorine was used a solution of 10 mg/L sodium hypochlorite prepared
229 from commercial bleach with a concentration of sodium hypochlorite equivalent to 5% (50
230 g/L). Bacterial suspensions (A610 = 0.1) prepared in saline solution were exposed to
231 sodium hypochlorite at a final concentration of 5 mg/L. A solution of 1.5% (w/v) sodium
232 thiosulfate was used to neutralize the effect of chlorine at different exposure times of: 0, 2,
233 7, 12, 17, 25 and 60 minutes. Cultures were plated immediately after the addition of the
234 neutralizing agent.

The effect of hydrogen peroxide was tested using a 0.1% solution prepared from a 30% stock (Carlo Erba Reagents, Italy). Bacterial suspensions (A610 = 0.1) were exposed to hydrogen peroxide at a final concentration of 0.05% (v/v). A freshly prepared solution of bovine liver catalase (0.1 g/L) was used in a ratio 0.1/5 (v/v) to eliminate residual hydrogen peroxide (Fiorentino et al. 2015) after exposure times of: 0, 2, 7, 12, 17, 25 and 60 minutes. Cultures were plated after catalase addition.

Statistical analyses

The effect of different stressors and the behavior of different strains was compared based on the parametric test one way ANOVA or the nonparametric tests Kruskal-Wallis and Mann-Whitney, depending if the results followed or not a normal distribution. The capacity to form biofilm in the presence and absence of stressors was compared based on the nonparametric test Mann-Whitney. The effect of disinfectants on cells inactivation was compared based on parametric one way ANOVA test with post-hoc test Tuckey. All the statistical analyzes were performed with the SPSS software package, version 23.0 (IBM SPSS software, Chicago, IL).

RESULTS

Ralstonia spp. tolerance to antibiotics and heavy metals

Based on the 16S rRNA gene sequence analyses, the *R. pickettii* and *R. mannitolilytica* strains studied clustered together with others from sources such as plant/animal, clinical/human, water and soil, or other environments (e.g. air) (**Fig. 1**). Although not

related to the isolation origin, three phylogenetic subgroups could be distinguished, one that included strains H2Cu2, L1PA1 and T6BT1, sharing a 16S rRNA gene sequence identity of 99.7 - 99.9%, other including strain H2Cu5, with a 16S rRNA identity with first group of 99.0 - 99.2% and another one of *R. mannitolilytica*, which, non-surprisingly included the strain T6BT10 with a 16S rRNA gene sequence identity of 97.8 - 98.2% with the *R. pickettii* isolates tested. These differences were not confirmed at the biochemical phenotype for which the five strains displayed a similar profile (data not shown).

All strains were observed to be resistant to colistin and ticarcillin and susceptible to the quinolones (nalidixic acid, ciprofloxacin), sulfonamides (sulfamethoxazole and sulfamethoxazole/trimethoprim), beta-lactams (cephalothin and ceftazidime) and tetracycline. Variable phenotypes were observed for meropenem and aminoglycosides susceptibility. Strains *R. pickettii* H2Cu5 and *R. mannitolilytica* T6BT10, both susceptible to gentamicin, differed on the susceptibility to streptomycin observed only in *R. mannitolilytica* T6BT10. Hence, gentamicin resistance was observed in the group of phylogenetically closely related strains H2Cu2, L1PA1 and T6BT1, with MIC-gentamicin values >256 mg/L, while strains H2Cu5 and T6BT10, in distinct phylogenetic subgroups, presented lower MIC values for both gentamicin and streptomycin (Table 2). MIC values for As^{3+} were about 30 times higher in gentamicin resistant than in gentamicin susceptible isolates, while no differences among strains were observed for Ni^{2+}or Cu^{2+}. This finding suggested that aminoglycoside and arsenite resistance mechanisms might be associated.

Stressors and growth kinetic

277 Based on the hypothesis that a common mechanism of resistance could be used by these
278 strains for gentamicin and arsenite, growth kinetic parameters were determined in the
279 absence and in the presence of each of those stressors (Table 3). In the absence of any
280 stressor, the growth rates for the five strains were similar (~0.4 h^{-1}) (Table 3). Either
281 gentamicin or arsenite led to significant ($p < 0.05$) reductions in the growth rate, being the
282 highest reductions observed in the presence of sub-inhibitory concentrations of gentamicin
283 (Table 3). The lag phases in the absence of stressor ranged 0.7-0.9 h. In the presence of
284 arsenite, but not in the presence of gentamicin, these values significantly ($p < 0.05$)
285 increased (to 2.4-3.4 h) in the strains with highest MIC-As^{3+} values (Table 3). In absence of
286 stressors, growth yield ranged 2.4-2.9. These values that were significantly ($p < 0.05$)
287 reduced in the presence of arsenite for strain H2Cu2 (to 2.2) or in the presence of
288 gentamicin for strains H2Cu2, H2Cu5, L1PA1, and T6BT1 (to 0.5-0.9). In general, the
289 reduction of growth yield was more pronounced in the presence of gentamicin than of
290 arsenite. These differences in the growth parameters in the presence of gentamicin or
291 arsenite suggest that even if a common resistance mechanism is used to grow in the
292 presence of each of those stressors, probably distinct functions are targeted in the cell by
293 the antibiotic or the metal.

294 Effect of stressors in the capacity of biofilm formation

295 The capacity to form biofilm may be an advantage in *Ralstonia* spp. to face adverse
296 conditions (Adley et al. 2005; Anderson et al. 1990; Di Domenico et al. 2016; Ryan et al.
297 2011). Hence, it was hypothesized that the stressors aminoglycosides and arsenite could
298 stimulate the capacity to produce biofilm (**Fig. 2**). The low concentrations of stressor
299 tolerated by the aminoglycoside susceptible strains H2Cu5 and T6BT10 were not observed

300 to induce in those strains an increased capacity to form biofilm. In contrast, the strains
301 resistant to the aminoglycosides gentamicin and streptomycin (H2Cu2, T6BT1 and L1PA1)
302 presented significant increases in the capacity to form biofilm, with increases of 2-4 times
303 for the lower concentrations and 5-11 times for the highest concentrations of
304 aminoglycosides tested, in comparison with the non-stressor assays (**Fig. 2**). In the same
305 way, 1.1 mmol/L arsenite a significant, although lower (1.5-1.9 times), increase of biofilm
306 formation. The capacity to form biofilm can be associated with the production of
307 polysaccharide capsules that facilitate the adherence to surfaces and the formation of
308 biofilms (Moxon and Kroll 1990). It was thus hypothesized that the increased capacity to
309 form biofilm could be due to an observable overproduction of capsule polysaccharides in
310 the presence of sub-inhibitory concentration of gentamicin. However, this hypothesis was
311 not proved, eventually because the method used to observe capsules was not sufficiently
312 sensitive.

313 Disinfectants and inactivation

314 The hypothesis beyond these assays was that aminoglycoside and arsenite resistant strains
315 would present a higher resilience against the different types of disinfectant - UV radiation,
316 chlorine, and peroxide disinfection (**Fig. 3**). However, it was observed that only peroxide
317 disinfection supported that hypothesis. Neither UV radiation nor chlorine were observed to
318 produce a distinct effect on the gentamicin resistant or susceptible strains (**Fig. 3**). The UV
319 radiation promoted a reduction of 1-2 log at each 15 min of exposure till the maximum
320 period tested of 45 min (**Fig. 3A**). In the presence of 5 mg/L chlorine it was observed a
321 sharp culture inactivation (2 min), to reach after 7 min of exposure, counts < 10 CFU/mL
322 (Log 1) (**Fig. 3B**). In contrast to the other two disinfectants, hydrogen peroxide revealed

higher antibacterial effectiveness against the gentamicin and arsenite susceptible strains than against the resistant. Susceptible strains decreased to counts below the quantification limit (one log-unit) after 7 min of exposure, in contrast to the resistant strains that required 12 min to reach < one log-unit (**Fig. 3C**).

DISCUSSION

Ralstonia spp. are ubiquitous, mainly in aquatic environments, including drinking water (Vaz-Moreira et al. 2017), and have been reported as contaminants of clinical sterile solutions or materials (Boutros et al. 2002; Labarca et al. 1999; McNeil et al. 1985) or as the prevalent species in hemodialysis water samples (Vincenti et al. 2014). *Ralstonia pickettii* strains from different clinical and environmental origins were reported as being resistant to gentamicin, ticarcillin and meropenem, although highly susceptible to ciprofloxacin, tetracycline and sulfamethoxazole/trimethoprim (Ryan and Adley 2013), confirming the phenotypes of the strains examined in this study. The observation that phylogenetically and phenotypically close *Ralstonia* strains, isolated from distinct aquatic environments, differed on the susceptibility to gentamicin (Table 1 and 2), suggested that it might be due to gene acquisition. Indeed, in a parallel study, whole genome sequencing from strains H2Cu2 and H2Cu5 showed that only the aminoglycoside resistant isolate contains genes associated with resistance to arsenic, and toxic compounds, encoding lysozyme inhibitors, or phages/prophages receptors (Vaz-Moreira et al. 2016).

The correlation observed between the resistance to arsenite and gentamicin, may also be an indication of a possible mechanism of co-resistance (genetic linkage between two or more

345 resistance genes) or cross-resistance (same genetic determinant confers resistance to both
346 antibiotics and metals), frequently reported for heavy metals and antibiotics (Baker-Austin
347 et al. 2006; Dib et al. 2008; Seiler and Berendonk 2012; Zhou et al. 2015) specially in
348 contaminated environments (Ahemad and Malik 2013).
349 Supposedly the physiological response to arsenite and aminoglycosides involves diverse
350 mechanisms, as the response for biofilm formation and growth kinetic did not respond in
351 the same mode for both antimicrobials (Table 2). However, the stress imposed by sub-
352 inhibitory concentrations of aminoglycosides or of metals increased the capacity to form
353 biofilms of the aminoglycoside resistant isolates (**Fig. 2**). This can be due to an unspecific
354 stress response, not related with the resistance mechanism, in particular enhanced
355 production of extracellular polymeric substances (EPS) which lead to cell adhesion (Baker-
356 Austin et al. 2006; Donlan 2002; Donlan and Costerton 2002; Lindsay and von Holy 2006),
357 and biofilm formation (Balaban 2008; Donlan and Costerton 2002; Lindsay and von Holy
358 2006). Similar results were observed for *Pseudomonas aeruginosa* and *Escherichia coli*
359 isolates, increasing their capacity to form biofilm in the presence of aminoglycosides (Aka
360 and Haji 2015; Hoffman et al. 2005). However, Paul *et al.* (2014) observed a negative
361 influence of 0.01 mmol/L arsenite on the capacity to form biofilm of *Pseudomonas* spp. or
362 *Rhizobium* spp. strains. Some authors refer to the importance of the bacteria motility on the
363 capacity to form biofilm (Guttenplan and Kearns 2013; O'Toole and Kolter 1998; Yao and
364 Allen 2007). Indeed, *Ralstonia* spp. are motile and this property might influence the
365 variable capacity to form biofilm observed in the present study. However, it was not
366 possible to assess differences in motility capabilities in the tested strains or to investigate if
367 stressors interfere with flagella and therefor with biofilm formation. Considering the history

of *Ralstonia* spp. as important colonizers of highly oligotrophic environments, other conditions to explore could be the effect of nutritional stress.

In contrast to what was observed for hydrogen peroxide, the survival to UV radiation or chlorine disinfection did not differ in aminoglycoside resistant or susceptible strains (**Fig. 3**). This observation is probably related with the inactivation mechanisms involved, DNA/RNA damage for UV, cell metabolism for chlorine and oxidative stress for hydrogen peroxide (Estrela et al. 2002; Hijnen et al. 2006; McDonnell and Russell 1999). These results suggest that the mechanisms involved in the aminoglycoside resistance are probably not directly associated with the mechanisms of survival to UV radiation or chlorination. Studies performed with a higher number of isolates, from different origins may give more consistency to these results. The capability of *Ralstonia* spp. to survive in some environments subjected to disinfection processes, as for example the wastewater or drinking water systems, is probably determinant for their capacity to spread or be transmitted to humans. It is curious to note that aminoglycoside resistant strains have increased capacity to form biofilm in the presence of some environmental stressors, since this may be a relevant factor to facilitate the survival and spread of aminoglycoside resistant *Ralstonia* spp. strains in the environments subjected to stress conditions, as antimicrobial challenges. These characteristics combined with the oligotrophic character may contribute for the *Ralstonia* ubiquity in aquatic habitats.

CONCLUSIONS

The aminoglycosides resistance was associated with the highest tolerance to arsenite.

Sub-inhibitory concentrations of gentamicin or arsenite significantly decreased the growth rate and yield, while arsenite but not gentamicin caused a significant increase of the lag phase. The biofilm formation was stimulated in the presence of aminoglycosides or arsenite, in the aminoglycoside resistant but not in the susceptible strains.

Disinfection with UV or chlorine presented identical effectiveness in aminoglycoside resistant or susceptible strains. In contrast, hydrogen peroxide presented higher effectiveness against aminoglycoside susceptible than resistant strains.

The results support the hypothesis that antibiotic resistance is associated with improved tolerance to stress.

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579

580 **TABLES**:

581 Table 1. Group of isolates tested in this study

582

Strain	Species	Source of isolation	Isolation medium and conditions	Abundance in the source (order of magnitude, CFU's/mL)	Reference
H2Cu2	*R. pickettii*	Hospital wastewater	Culture enrichment in modified Luria-Bertani broth with Cu^{2+} (2.5 mmol/L)	10^3	Becerra-Castro *et al*. 2015
H2Cu5	*R. pickettii*	Hospital wastewater	Culture enrichment in modified Luria-Bertani broth with Cu^{2+} (2.5 mmol/L)	10^3	Becerra-Castro *et al*. 2015
L1PA1	*R. pickettii*	Mineral water	Pseudomonas isolation agar with 32mg/L amoxicillin	10^1	Falcone-Dias *et al*. 2012
T6BT1	*R. pickettii*	Tap water	Tergitol 7-agar	10^{-1}	Vaz-Moreira *et al*. 2013
T6BT10	*R. mannitolilytica*	Tap water	Tergitol 7-agar	10^{-1}	Vaz-Moreira *et al*. 2013

583 Table 2. Minimum Inhibitory Concentrations (MICs) for antibiotics and metals determined

584 for the *Ralstonia* spp. strains under study.

Strain	MICs								
	GEN (mg/L)	**STR (mg/L)**	**TET (mg/L)**	**MER (mg/L)**	**CEF (mg/L)**	**SUL (mg/L)**	**As^{3+} (mmol/L)**	**Ni^{2+} (mmol/L)**	**Cu^{2+} (mmol/L)**
H2Cu2	> 256	>1024	1	>32	6	24	1.4	4	12
H2Cu5	6	56	0.25	6	6	4	0.05	4	12
L1PA1	> 256	>1024	1	>32	6	24	1.4	4	12
T6BT1	> 256	>1024	1	16	8	24	1.4	4	12
T6BT10	0.5	4	8	>32	4	4	0.05	4	12

585 GEN, gentamicin; TET, tetracycline; MER, meropenem; CEF, ceftazidime; SUL, sulfamethoxazole; STR,

586 streptomycin and metal salts of As^{3+}, Ni^{2+} and Cu^{2+}

Table 3. Variations on the bacterial growth parameters growth rate, phase lag and yield, under sub-inhibitory concentrations of arsenite (As^{3+}) or gentamicin (GEN) or control conditions (stressor free, SF).

Strain (stressor concentration)	**Growth rate (per hour)**			**Phase Lag (hours)**			**Yield (A610)**		
	SF	**As^{3+}**	**GEN**	**SF**	**As^{3+}**	**GEN**	**SF**	**As^{3+}**	**GEN**
H2Cu2 125 mg/L GEN or 1.1 mmol/L As^{3+}	1;a,b 0.4±0.03	2;a 0.3±0.02	3;a 0.1±0.01	1;a 0.9±0.2	2;a 3.4±0.5	1,2;a 2.0±0.7	1;a 2.9±0.1	2;a 2.2±0.2	3;a 0.5±0.1
H2Cu5 6 mg/L GEN or 0.01 mmol/L As^{3+}	1;a 0.3±0.01	1;a 0.3±0.04	2;b 0.1±0.01	1;a 0.7±0.4	1;b 1.0±0.3	1;a 2.2±2.5	1;a 2.5±0.3	1;a 2.0±0.3	2;a 0.9±0.2
L1PA1 125 mg/L GEN or 1.1 mmol/L As^{3+}	1;a,b 0.4±0.04	1;a,b 0.3±0.04	2;c 0.2±0.01	1;a 0.7±0.4	2;a 3.0±0.9	1;a 0.8±0.1	1;a 2.9±0.4	1;a 2.5±0.5	2;a 0.8±0.1
T6BT1 125 mg/L GEN or 1.1 mmol/L As^{3+}	1;a,b 0.4±0.02	2;a 0.3±0.01	3;b 0.2±0.01	1;a 0.9±0.3	2;a 2.4±0.3	1;a 1.4±0.4	1;a 2.7±0.1	1;a 2.4±0.3	2;a 0.7±0.1
T6BT10 0.4 mg/L GEN or 0.01 mmol/L As^{3+}	1;b 0.4±0.01	1,2;b 0.4±0.01	2;d 0.4±0.01	1;a 0.8±0.1	1;b 0.8±0.1	1;a 0.7±0.1	1;a 2.4±0.5	1;a 2.1±0.4	1;b 1.9±0.6

A610, bacterial suspension absorbance at 610 nm;

Statistically significant differences between stress conditions (SF, As^{3+} and GEN) are indicated by the numbers: 1, 2, 3; and significant differences between strains are indicated by the letters: a, b, c, d.

FIGURES:

Fig. 1 Environmental distribution of *Ralstonia pickettii* and *Ralstonia mannitolilytica*, including the strains studied (in red).

Fig. 2 Influence of stressors on the ability of biofilm formation, for the strains H2Cu2 (A), T6BT1 (B) and L1PA1 (C). The quantification of the biofilm formation was performed through the calculation of a ratio A_{570}/A_{620}, referring to absorbance at 570 nm (measure of the biofilm formation) and absorbance at 620 nm (measure of the bacterial growth). The non-inoculated control presented a ratio A_{570}/A_{620} of 1.0±0.1; and the *P. aeruginosa* presented ratios A_{570}/A_{620} of: 3.1±0.7 for stressor-free (SF); 2.2±0.9 for gentamicin (GEN) 6 mg/L; 2.8±0.7 for streptomycin (STR) 50 mg/L; 0.2±0.09 for meropenem 4 mg/L; 1.9±0.9 for arsenite (As) 0.01 mmol/L; 0.3±0.05 for copper 6.0 mmol/L; and 0.7±0.2 for nickel 2.5 mmol/L.

Fig. 3 Bacterial inactivation with A) UV radiation, B) chlorine (5 mg/L), and C) hydrogen peroxide (0.05%).