CATÓLICA
ESCOLA SUPERIOR DE BIOTECNOLOGIA
PORTO

Ecology and antimicrobial resistance of *Ralstonia* spp. in the urban water cycle

Thesis submitted to the *Universidade Católica Portuguesa* to attain the degree of PhD in Biotechnology with specialization in Microbiology

Félix Pompeyo Ferro Mayhua

June 2019

CATÓLICA
ESCOLA SUPERIOR DE BIOTECNOLOGIA
PORTO

Ecology and antimicrobial resistance of *Ralstonia* spp. in the urban water cycle

Thesis submitted to the *Universidade Católica Portuguesa* to attain the degree of PhD in Biotechnology with specialization in Microbiology

Félix Pompeyo Ferro Mayhua

Supervisor: Professor Célia Maria Manaia Rodrigues

Co-supervisor: Dra. Ivone Cristina Vaz Moreira

June 2019

“Water is the driving force of all nature”

Leonardo Da Vinci.

to all the people Quechua

Resumo

As Betaproteobactérias são ubíquas no ciclo urbano da água, com múltiplas oportunidades de interacção com os seres humanos. A combinação das propriedades fisiológicas e ecológicas de algumas Betaproteobactérias explica sua capacidade de sobreviver no meio ambiente, persistir após desinfecção da água potável e, por vezes, colonizar animais, incluindo humanos. Versatilidade metabólica, capacidade de formar biofilme e tolerância a agentes antimicrobianos e metais, são exemplos de características subjacentes à ubiquidade acima mencionada. Porém, esta informação não se encontra claramente disponível na literatura científica, pelo que um dos objectivos deste trabalho, foi o de investigar e sistematizar informação sobre a ocorrência de Betaproteobactérias em água potável. Três géneros bacterianos de *Betaproteobacteria*, cuja presença em água exemplifica a referida ubiquidade são *Achromobacter, Burkholderia* e *Ralstonia*, por vezes relacionados a surtos de infecção associados a água potável. *Ralstonia* spp. despertou particular atenção visto ter sido recentemente isolada no grupo de fontes tão diversas como água mineral ou água de esgoto hospitalar. *Ralstonia* spp., principalmente as espécies *Ralstonia pickettii* e *R. mannitolilytica*, têm sido recentemente associadas casos de infecção e esta ubiquidade suscitou interesse. Membros destas espécies podem apresentar tolerância a metais, antibióticos e desinfetantes, o que pode contribuir para a sua ubiquidade e eventual severidade como agentes infecciosos. Neste estudo procurou compreender-se se existia uma inter-relação entre resistência a metais e antibióticos e como é que tal poderia influenciar o comportamento das estirpes. O trabalho experimental envolveu a caracterização de estirpes das espécies *R. pickettii* e *R. mannitolilytica*, isoladas de água de esgoto hospitalar, água da torneira e de água mineral engarrafada, e teve como objectivo identificar possíveis associações de fenótipos de resistência a antibióticos e metais e outros tipos de stresse, bem como as suas bases genéticas. Especificamente, num primeiro ensaio estudaram-se isolados de água de *Ralstonia pickettii* e *R. mannitolilytica* de efluente hospitalar (n = 2), água da torneira (n = 2), água mineral (n = 1), e avaliaram-se os fenótipos de resistência a antibióticos e a metais bem como resposta a desinfecção e alguns parâmetros de cinética de crescimento. Para atingir esses objetivos, utilizou-se o método de difusão em disco e de microdiluição para determinar concentrações inibitórias mínimas (MIC), fizeram-se curvas de crescimento em meio Mueller-Hinton suplementado com os agentes antimicrobianos de interesse, de modo a estudar os parâmetros de cinética de crescimento, avaliou-se a capacidade de

formação de biofilme e testou-se o comportamento em presença de radiação ultravioleta, hipoclorito de sódio e peróxido de hidrogénio. Este estudo sugeriu existir uma associação entre resistência a gentamicina (MIC > 256 mg/L) e tolerância aumentada a arsenito (MIC = 1.4 mmol/L) A hipótese de se tratar de resistência cruzada foi descartada visto, concentrações sub-inibitórias de gentamicina ou de arsenito diminuíram significativamente a taxa de crescimento e produção de *Ralstonia* spp. embora apenas o arsenito causasse um aumento significativo na fase de lag. Além disso, em estirpes resistentes a gentamicina, a formação de biofilme foi estimulada na presença de aminoglicosídeos ou de arsenito. A desinfecção com radiação ultravioleta ou hipoclorito apresentou taxas de inativação semelhantes nas linhagens resistentes e suscetíveis a gentamicina. Em contraste, a mesma dose de peróxido de hidrogénio causou inativação mais rápida em estirpes sensíveis a gentamicina do que em resistentes.

A associação entre resistência a gentamicina e arsenito foi explorada num grupo mais alargado de *R. pickettii*, pelo que se incluíram 37 estirpes de, 14 de água mineral, 17 de água de torneira e 6 de esgoto hospitalar. Analisaram-se os perfis de resistência a antibióticos e a arsenito, conforme se descreve acima e fez-se o rastreio de determinantes genéticos de resistência selecionados, incluindo os relacionados com Elementos Conjugativos Integrativos (ICE), plasmídeos, e genes associados com uma bomba de efluxo e resistência ao arsénio. A maioria dos isolados (32/37) era resistente a gentamicina, beta-lactâmicos e colistina. Porém, nem todos os isolados eram resistentes a gentamicina e a associação deste fenótipo com o aumento da tolerância ao arsenito foi confirmada. Esta divisão de estirpes resistentes a gentamicina e arsenito *vs*. suscetíveis concidia com a separação de dois grupos filogenéticos, com base na análise do gene rRNA 16S. Os isolados resistentes a gentamicina e arsenito apresentaram ICEs e os genes *arsH* e *acr3*, relacionados à resistência ao arsenito. A maioria dos isolados de *R. pickettii* (36) apresentava um ou dois plasmídeos com tamanhos entre 77 e 260 kpb. As sequências de aminoácidos da bomba de efluxo, cujo gene amplificou em todas as estirpes, diferiam em isolados resistentes e suscetíveis. Embora os genótipos de estirpes resistentes e suscetíveis a gentamicina diferissem de forma consistente com os fenótipos e com a distinção filogenética de ambos os grupos, não foi possível encontrar uma explicação genética para o fenótipo observado, nem para a associação de resistência arsenito e gentamicina. Porém, para todos os elementos genéticos analisados verificou-se uma divisão clara entre ambos os grupos, sugerindo que abordagens de genómica comparativa poderiam contribuir para

elucidar as bases genéticas dos fenótipos em análise – resistência vs. suscetibilidade a gentamicina e arsenito. Neste sentido, compararam-se os genomas de uma estirpe resistente e de uma estirpe suscetível e com especial enfoque nas categorias funcionais "Transporte Membranar", "Stress e Defesa" e "Virulência" analisaram-se os genes presentes apenas na estirpe resistente ou as mutações detetadas entre ambos. Esta análise mostrou haver algumas funções que podem explicar quer a capacidade para adquirir ou desenvolver novos mecanismos de resistência. Este tipo de estudo permite elucidar os processos de desenvolvimento ou aquisição de resistência em espécies ubíquas caracterizadas por mecanismos de resistência assumidamente intrínseca, visto serem comuns à maioria de membros de uma espécie.

Palavras-chave: Bactérias ubíquas; Patogénicos oportunistas; Tolerância ao stresse; Resistência intrínseca; Resistência adquirida.

Abstract

Betaproteobacteria are ubiquitous in the urban water cycle, with multiple opportunities for interaction with humans. The combination of the physiological and ecological properties of some *Betaproteobacteria* explains their ability to survive in the environment, persist after disinfection of drinking water and sometimes colonize animals, including humans. Metabolic versatility, ability to form biofilm and tolerance to antimicrobial agents and metals, are examples of characteristics underlying the aforementioned ubiquity. However, this information is not clearly available in the scientific literature, so one of the objectives of this work was to investigate and systematize information on the occurrence of *Betaproteobacteria* in drinking water. Three bacterial genera of *Betaproteobacteria*, whose presence in water exemplifies the mentioned ubiquity are *Achromobacter*, *Burkholderia* and *Ralstonia*, sometimes related to outbreaks of infection associated with drinking water. *Ralstonia* spp. aroused particular attention since it was recently isolated from a group of sources as diverse as mineral water or hospital sewage. *Ralstonia* spp., mainly the species *Ralstonia pickettii* and *R. mannitolilytica*, have recently been associated with cases of infection and this ubiquity has aroused interest. Members of these species may tolerate metals, antibiotics and disinfectants, which may contribute to their ubiquity and eventual severity as infectious agents. In this study it was sought to understand if there was an interrelation between resistance to metals and antibiotics and how this could influence the behavior of the strains. The experimental work involved the characterization of strains of the *R. pickettii* and *R. mannitolilytica* species, isolated from hospital sewage, tap water and bottled mineral water, and aimed to identify possible associations of resistance phenotypes to antibiotics and metals and other types of stress, as well as their genetic basis. Specifically, in a first trial, water isolates of *R. pickettii* and *R. mannitolilytica* from hospital effluent (n = 2), tap water (n = 2), mineral water (n = 1) were studied, and resistance phenotypes to antibiotics and metals as well as disinfection response and some parameters of growth kinetics were evaluated. To achieve these objectives, the disc diffusion and microdilution method was used to determine minimum inhibitory concentrations (MICs), growth curves were made in Mueller-Hinton broth culture medium supplemented with the antimicrobial agents of interest, in order to study the parameters of growth kinetics, the biofilm formation capacity was evaluated and the behavior was tested in the presence of ultraviolet radiation, sodium hypochlorite and hydrogen peroxide. This study suggested

an association between resistance to gentamicin (MIC > 256 mg/L) and increased tolerance to arsenite (MIC = 1.4 mmol/L). The cross-resistance hypothesis was ruled out since sub-inhibitory concentrations of gentamicin or arsenite significantly decreased the growth rate and yield of *Ralstonia* spp., although only the arsenite caused a significant increase in the lag phase. In addition, in strains resistant to gentamicin, biofilm formation was stimulated in the presence of aminoglycosides or arsenite. Ultraviolet or hypochlorite disinfection exhibited similar inactivation rates in resistant and susceptible gentamicin strains. In contrast, the same dose of hydrogen peroxide caused faster inactivation in strains sensitive to gentamicin than in resistant strains.

The association between resistance to gentamicin and arsenite was explored in a larger group of *R. pickettii.* A group of 37 strains, 14 from mineral water, 17 from tap water and 6 from sewage hospital were included. The antibiotic and arsenite resistance profiles were screened as described above and selected resistance genetic determinants, including those relating to Integrative and Conjugative Elements (ICEs), plasmids, and genes associated with efflux and arsenic resistance were analyzed. Most of the isolates (32/37) were resistant to gentamicin, beta-lactam and colistin. However, not all isolates were resistant to gentamicin and the association of this phenotype with increased arsenite tolerance was confirmed. This division of susceptible vs. resistant strains to gentamicin and arsenite was consistent with the separation of two phylogenetic groups, based on analysis of the 16S rRNA gene. Moreover, isolates resistant to gentamicin and arsenite showed ICEs and the *arsH* and *acr3* genes, related to the resistance to arsenite. Most of the *R. pickettii* isolates (36) had one or two plasmids with sizes between 77 and 260 kbp. The deduced amino acid sequences of the efflux pump, whose gene amplified in all strains, differed in resistant and susceptible isolates. Although genotypes of resistant strains susceptible to gentamicin differed in a manner consistent with the phenotypes and with the phylogenetic distinction of both groups, it was not possible to find a genetic explanation for the observed phenotype nor for the association of arsenite and gentamicin resistance. However, for all the analyzed genetic elements there was a clear division between both groups, suggesting that approaches of comparative genomics could contribute to elucidate the genetic bases of the phenotypes under analysis - resistance vs. susceptibility to gentamicin and arsenite. In this sense, we compared the genomes of one resistant and one susceptible strain, and with special focus on the functional categories "Membrane transport", "Stress and response" and "Virulence, disease and defense" were analyzed the

genes present only in the resistant strain or mutations detected between both. This analysis has shown some functions that may explain either the ability to acquire or develop new resistance mechanisms. This type of study allows elucidating the processes of development or acquisition of resistance in ubiquitous species characterized by mechanisms of intrinsic resistance, since they are common to the majority of members of a species.

Keywords: Ubiquitous bacteria; Opportunistic pathogens; Stress tolerance; Intrinsic resistance; Acquired resistan.

Acknowledgments

A la Escuela Superior de Biotecnologia (ESB) de la Universidad Catolica Portuguesa por haberme aceptado como estudiante del doctorado y por proveerme todas las condiciones necesarias para realizar el presente trabajo de investigacion.

A mi supervisora Profesora Celia Manaia y a mi co-supervisora Dra. Ivone Vaz-Moreira, por haberme aceptado como doctorando, por su dedicacion y adecuada orientacion para la ejecucion y culminar satisfactoriamente esta tesis, tambien por su paciencia y compartir sus conocimientos.

A todos los profesores de la ESB, mi sincero agradecimiento.

A mi familia, que a pesar de estar lejos, siempre me alentaron desde el "nuevo" continente.

Mi sincero agradecimiento a la Red de Salud Chucuito, Región de Salud Puno del Ministerio de Salud del Perú, por la licencia de estudios.

Finalmente mi agradecimiento a Erasmus Mundus Project SUD-EU por el financiamiento respectivo (EMA2-STRAND 1, LOT 14) y al CONCYTEC Perú a través de su unidad ejecutora: FONDECYT, por el financiamiento para completar mi propósito: la defensa de la tesis.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

AK	Amikacin
CEF	Ceftazidime
CIP	Ciprofloxacin
CP	Cephalothin
CT	Colistin sulfate
GEN	Gentamicin
GR	Gentamicn resistant
GS	Gentamicin susceptible
HGT	Horizontal gene transfer
ICEs	Integrative and Conjugative Elements
K	Kanamycin
MDR	Multidrug resistance
MGEs	Mobile genetic elements
MICs	Minimum inhibitory concentrations
MER	Meropenem
NA	Nalidixic acid
NET	Netilmicin
OM	Outer membrane
PCA	Plate Count Agar
QS	Quorum-sensing
rRNA	Ribosomal ribonucleic acid.
STR	Streptomycin

SUL	Sulfamethoxazole
SXT	Sulfamethoxazole/trimethoprim
TET	Tetracycline
TIC	Ticarcillin
TOB	Tobramycin
UV	Ultraviolet

Publications

The content of this thesis was partially published:

Ferro, Pompeyo, Vaz-Moreira, Ivone, and Manaia, Celia M. 2019. "Association between Gentamicin Resistance and Stress Tolerance in Water Isolates of *Ralstonia pickettii* and *R. mannitolilytica*." Folia Microbiologica 64.1:63-72. DOI: 10.1007/s12223-018-0632-1

Ferro, Pompeyo, Vaz-Moreira, Ivone and Manaia, Celia M. "*Betaproteobacteria* are predominant in drinking water: are there reasons for concern?". Critical Reviews in Microbiology (under review).

Ferro, Pompeyo, Vaz-Moreira, Ivone and Manaia, Celia M. "Hints for acquired gentamicin and arsenite resistance in *Ralstonia pickettii* water isolates". In preparation

1. Introduction

Water habitats are among the most important bacterial niches in nature. Aquifers, groundwater, lakes, rivers, wastewater and drinking water house a high number and diverse group of bacterial lineages (Tamames *et al.,* 2010). Water is also an important vehicle of dissemination of microorganisms in nature (Baquero *et al.,* 2008; Rizzo *et al.,* 2013; Xi *et al.,* 2009; Zhang *et al.,* 2009). The urban water cycle is in this aspect particularly relevant, since it combines different stages in which water is impacted by or can impact human activities and health. This cycle comprises the different stages from the water collection for drinking water production till the delivery, after use, to a natural water body (e.g. a river). Processes such as purification, transport, storage, distribution, and consumption, after which sewerage and wastewater treatment, as well as intended or unintended water reuse, are all part of this cycle (Zhang *et al.,* 2016; Calero-Caceres *et al.,* 2017; Gao *et al.,* 2017; Marsalek and Jimenez, 2006; Ramoa *et al.,* 2015; SWITCH, 2010; Vaz-Moreira *et al.,* 2014; Yang *et al.,* 2017).

1.1. Bacterial diversity in the urban water cycle

Members of the phyla *Proteobacteria* (mainly the classes *Alpha*- and *Betaproteobacteria), Actinobacteria, Bacteroidetes* and *Firmicutes* are among the most common in drinking water (Vaz-Moreira *et al.,* 2017; Vaz-Moreira *et al.,* 2014). Some genera and species of these groups are poorly characterized and their ecology and potential human health impacts deserve further studies. One of such groups are members of the genus *Ralstonia* of the class *Betaproteobacteria*, which can be found in aquatic habitats, either pristine, disinfected or polluted (Becerra-Castro *et al.,* 2015; Falcone-Dias *et al.,* 2012; Vaz-Moreira *et al.,* 2014). This fact, combined with the physiological versatility, motivated the selection of this genus for this research work.

1.2. The ubiquity of *Ralstonia* spp.

The genus *Ralstonia*, established in 1995 (Yabuuchi *et al.,* 1995), includes species formerly designated as members of the genera *Burkholderia* (*Burkholderia pickettii* and *Burkholderia solanacearum*) and *Pseudomonas* (*Pseudomonas pickettii*), renamed as *Ralstonia pickettii* and *Rastonia solanacearum*, respectively. The reclassification was supported by distinct phenotypic characteristics and 16S rRNA gene based phylogenetic analyses (Daxboeck *et al.,* 2005; Ryan *et al.,* 2006). Currently the genus *Ralstonia* comprises 6 species (*R. insidiosa*, *R. mannitolilytica, R. solanacearum*, *R.pickettii*, *R. pseudosolanacearum*, and *R. syzygii*), with *R. pickettii* as the type species of the genus (Parte, 2019).

The aquatic habitats where *Ralstonia* spp., mainly *R. pickettii* and *R. mannitolilytica.* have been described (Table 1.1) include wastewater, potable water, surface water, mineral water, and highly purified water (Adley *et al.,* 2005; Becerra-Castro *et al.,* 2015; Falcone-Dias *et al.,*2012; Kulakov *et al.,*2002; Ryan *et al.,*2006; Vaz-Moreira *et alle* 2014), or even the water system of the space shuttle (Koenig, 1997). *R. pickettii* was reported also in biofilms colonizing the interior of plastic (Poly Vinyl Chloride, PVC) pipes (Anderson *et al.,* 1990). In addition, *Ralstonia* spp. have also been reported in a wide variety of other habitats, including plants and soils contaminated with heavy metals (Coenye *et al.,* 2002), skin disinfectants (Maroye *et al.,* 2000), saline solutions used for patients care (Chen *et al.,* 2017; Labarca *et al.,* 1999; McNeil *et al.,* 1985), caps of blood culture bottles (Boutros *et al.,* 2002), blood, urine and cerebrospinal fluid (Riley and Weaver, 1975), the respiratory tract (Daxboeck *et al.,* 2005; Stelzmueller *et al.,* 2006), or the oral cavity (Stelzmueller *et al.,* 2006). This obvious ubiquity has been associated with the capacity of *R. pickettii* to grow in moist environments with minimal nutrient resources, from which it can be transmitted to humans, occasionally causing disease (Daxboeck *et al.,* 2005). Indeed, it has been suggested that *R. pickettii* finds its way into clinical environments mainly through contamination of accessories of water supplies (Ryan *et al.,* 2006), for instance, due to contamination during the manufacturing process (Daxboeck *et al.,* 2005) or handling accessories for water. The hypothesis that

difficulties in isolation and identification have contributed to disregard this opportunistic pathogen should not be neglected (Ryan *et al.,* 2006).

Table 1.1 Examples of *Ralstonia* spp. detected in aquatic environments.

Name	**Aquatic habitats**	**References**
Ralstonia spp. (mainly species *R. pickettii* ; *R. mannitolilytica* and *R. insidiosa*)	Wastewater	(Becerra-Castro *et al.,* 2015; Falcone-Dias *et al.,* 2012; Vaz-Moreira *et al.,* 2014)
	Tap or potable water	
	Surface water	
	Mineral water	
	Highly purified water	(Adley *et al.,* 2005)
	Water system of the space shuttle	(Koenig, 1997)
	Skin disinfectants	(Maroye *et al.,* 2000)
	Saline solutions	(Chen *et al.,* 2017; Labarca *et al.,* 1999; McNeil *et al.,* 1985)
	Caps of blood cultures bottles	(Boutros *et al.,* 2002)
	Biological samples (e.g. blood, urine, cerebrospinal fluid, respiratory tract)	(Verschraegen *et al.,* 1985)

1.3. Clinical relevance of the *Ralstonia* spp.

Although *Ralstonia* spp. are not considered primary pathogens and its virulence was believed to be low, *R. pickettii* and *R. mannitolilytica* have been associated with clinical episodes. Daxboeck *et al.,* (2005) argued that the statute of *R. pickettii* as a neglected pathogen must be re-evaluated in light of several reports of infection that identified the *R. pickettii* as the cause of potentially harmful infections, and even death (Ryan and Adley, 2014). Unusual and sometimes invasive and severe infections attributed to *R. pickettii* have been described, e.g. meningitis, septic arthritis and osteomyelitis (Ryan and Adley, 2013; Ryan *et al.,* 2006), in immunocompromised and cystic fibrosis patients (Stelzmueller *et al.,* 2006), in neonatal sepsis (Sharma *et al.,* 2017), also septic arthritis

(Zellweger *et al.,* 2004), with tonsillitis and wound infection (Abhishek *et al.,* 2018); in orthopedic contamination (Birlutiu *et al.,* 2017; Edwards *et al.,* 2017) and other occasional cases (CDC, 1998; Riley and Weaver, 1975; Waugh *et al.,* 2010). Also *R. mannitolilytica* has been associated with bacteremia episodes (Ryan and Adley, 2014), specifically in a neonatal intensive care unit (Camargo De Souza *et al.,* 2018), in a kidney transplant recipient (Boattini *et al.,* 2018), in a dialysis patient (Seong Lim and Lee, 2017); in cystic fibrosis (Coman *et al.,* 2017) or in septicemia in hospital outbreaks associated with contaminated treatment water or auxiliary instruments *(*Liu *et al.,* 2016). *R. insidiosa* have been also suggested as an emerging pathogen in intensive care units (Alasehir *et al.,* 2018). The low frequency of cases, moderate virulence and difficult diagnosis may explain the fact that these bacteria are not monitored in routine clinical analyses (Gilligan *et al.,* 2003).

1.4. Antibiotic resistance in environmental bacteria: the scant information for *Ralstonia* spp.

The evolution and spread of antibiotic resistance may be related to selective pressures present in the microbial environment. Selective effects may result from the environmental contamination with antibiotic residues, but other selective pressures may be due to the presence of heavy metals, xenobiotic compounds, and organic solvents that can contribute to the selection of antibiotic resistance genes (Alonso *et al.,* 2001; Steint 2011). Antibiotic resistant bacteria can have intrinsic properties and genetics determinants that are responsible for the capability to proliferate in the presence of an antibiotic. Others with acquired antibiotic resistance traits, gained such a capacity by mutation or horizontal gene transfer (Davies and Davies, 2010; Juhas *et al.,* 2009). Horizontal gene transfer (HGT) allows bacteria to acquire genetic material from other cells or from the environment and thereby can increase their biochemical capabilities. This is the main mechanism for the acquisition of genetic determinants of resistance to antibiotics, and this gene transfer may be carried out by three natural mechanisms: conjugation, transduction and transformation (Alanis, 2005; Madigan *et al.,* 2006). These processes may be subject to several selection processes in which mutation or acquisition

of different resistance genes represents an advantage. The consequences of these events can be enhanced by selection promoted by a single agent or by different antimicrobials exerting co-selection processes (Cantón and Ruiz-Garbajosa, 2011). Therefore, the environment has an important role in the selection of antibiotic resistance genes accelerating an adaptive response, a key element of the evolutionary process (Allen *et al.,* 2010; Alonso *et al.,* 2001). Most antibiotics are readily degraded in the environment, but not metals, which, therefore, could represent a long-term selective pressure, depending on the chemical form and bioavailability (Stepanauskas *et al.,* 2006).

Metals and antibiotic resistance can be associated in two different ways: a) co-resistance involving the presence of different resistance determinants in the same genetic element (plasmids, transposon or integron) (Baker-Austin *et al.,* 2006; Chapman, 2003); and b) cross-resistance, when the same genetic determinant encodes proteins or enzymes that are responsible for resistance to both antibiotics and metals; which can occur when different agents are the target for a single defense mechanism, normally extrusion, via efflux pumps (Baker-Austin *et al.,* 2006). Therefore, the presence of an antibiotic might select resistance to the same antibiotic and indirectly select resistance to other antibiotics and heavy metals (Baker-Austin *et al.,* 2006). For example, Ferreira da Silva *et al.,* (2007) suggested that mercury may act as a selective factor for the proliferation of antibiotic-resistant bacteria to tetracycline and sulfamethoxazole/trimethoprim. Acquired or intrinsic antibiotic resistance is propagated in the community via vertical inheritance, meaning the transmission of genetic material from mother cell to daughter cell during cell division (Lawrence, 2005). Vertical transmission is influenced by strain fitness and therefore it is important for the selection of resistance traits.

Ralstonia spp., mainly of the species *R. pickettii*, have been shown to yield a wide array of antibiotic resistance phenotypes, including resistance to gentamicin, chloramphenicol, colistin, tobramycin, polymyxin B, and many others (Ryan *et al.,* 2009). Isolates of *R. pickettii* recovered from patients with clinical manifestations of infection has shown resistance to different antibiotics: ciprofloxacin, sulphamethoxazole-trimethopim, aztreonam, piperacillin-tazobactam, ceftriaxone, ceftazidime, imipenem-cilastatine, and aminoglycosides (Stelzmueller *et al.,* 2006). Resistance phenotypes against antibiotics

such as amikacin, ceftriaxone, ciprofloxacin, mezlocillin, aztreonam, and gentamicin haves been noted in *R. pickettii* (Pan *et al.,* 2011). Similarly, clinical isolates of *R. mannitolilytica* can be resistant to gentamicin, amikacin and ceftazidime (Daxboeck *et al.,* 2005). In a recent study, selective pressures imposed by metals cooper and zinc, led to the isolation of cultures of metal resistant *R. pickettii* and *Elizabethkingia anophelis* (Becerra-Castro *et al.,* 2015). At the moment of writing not much is known regarding heavy metal or antibiotic resistance in *Ralstonia* spp. A recent contribution is the review of Ryan and Adley (2014), addressing the types of infection caused by *Ralstonia* spp. (*Ralstonia pickettii* and *R. mannitolilytic*a), and underlying conditions that are associated with these infections and potential treatments.

1.5. The cross talk between ecology and genome dynamics

Antibiotic resistant bacteria exhibit that property due to intrinsic characteristics or, on the contrary, due to the acquisition of genetic mutations or specific genetic elements that convert a once susceptible into a resistant organism. The intrinsic resistance to an antibiotic is the ability to resist the action of that antibiotic due to an inherent structural or functional characteristic (Blair *et al.,* 2014). According to Cox and Wright (2013), intrinsic resistance comprises defense mechanisms that are found in the cell naturally and it is a condition that is a common trait in bacterial species. The ability of microorganisms to adapt to different environments is largely due to the genes pool, capable of providing the capability to withstand different conditions (Aydin *et al.,* 2015; Pierro, 2015). Important reactors where antibiotic resistance can be selected include human and animal bodies, mainly in places under strong selective pressure, such as hospitals, long-term care facilities or farms; and some environmental compartments, such as sewage and wastewater treatment facilities, soil and sediments among other. In general, all these are reactors where bacteria and potential selective pressures can mix (Baquero *et al.,* 2008). Genes, including those conferring resistance, can be transferred among microorganisms by means of mobile genetic elements (MGEs) (Shintani and Nojiri, 2013). The acquisition of genes can be intracellular, such as the transfer of a gene from a plasmid to the chromosome or intercellular when contemporaneous cells deliver and receive genes. ICEs are self-transmissible MGEs that encode the machinery for conjugation as well as

intricate regulatory systems to control their excision from the chromosome and their conjugative transfer (Burrus and Waldor, 2004; Salyers *et al.,* 1995). These elements are able to integrate into and replicate as part of the host chromosome and may include genes associated with antibiotic resistance or virulence factors. ICEs can confer to the host selective advantages and therefore be a vital driving force for bacterial adaptation and evolution (Delavat *et al.,* 2017; Wozniak and Waldor, 2010), which is propagated passively during replication, segregation, and cell division (Johnson and Grossman, 2015). ICEs promote the vertical inheritance and sporadic lateral gene flow, spreading virulence and antibiotic-resistance determinants (Bi *et al.,* 2011), especially by interaction with other MGEs capable of HGT (Liu *et al.,* 2019). ICEs (such as Tn4371) were decribed by Ryan *et al.,* (2009) for *R. pickettii.*

1.6. Objectives

Ralstonia spp. are ubiquitous in water (Anderson *et al.,* 1990; Boutros *et al.,* 2002; Daxboeck *et al.,* 2005; Falcone-Dias *et al.,* 2012; Kulakov *et al.,* 2002; Ryan *et al.,* 2006; Vaz-Moreira *et al.,* 2013) and are able to withstand the drinking water treatment and may be consumed by humans. Considering the above background, and that the genetic resistance determinants present in *Ralstonia* spp., as well as the factors that may contribute for their persistence over wastewater and drinking water treatment and distribution, are almost unknown, this project aimed to assess the physiological and genetic diversity in *Ralstonia* spp., with emphasis on the genetic determinants and external variables that may be associated with antibiotic and stress tolerance. To reach this objective, it was i) reviewed the current state of the art regarding the occurrence of *Betaproteobacteria* in drinking water; ii) studied the association between gentamicin resistance and stress tolerance in water isolates of *R. pickettii* and *R. mannitolilytica*; iii) characterized the genetic resistance determinants, their stability and the factors that may trigger the mobilization and/or selection of antibiotic resistance in *R. pickettii.*

2. Thesis Roadmap

The phylum *Proteobacteria* is among the most abundant in drinking water (Vaz-Moreira *et al.,* 2014). Some of these bacteria yield intrinsic or acquired antibiotic resistance (Daxboeck *et al.,* 2005; Pan *et al.,* 2011; Ryan *et al.,* 2009; Stelzmueller *et al.,* 2006) and, in spite of the generally low pathogenicity, can behave as opportunistic pathogens (Coenye *et al.,* 2002). *Betaproteobacteria* class, in which is integrated the genus *Ralstonia*, comprise good examples of such a situation. This rationale motivated the interest in exploring the current state of the art concerning the taxonomic diversity and ecology of drinking water *Betaproteobacteria*, as well as what is known about their virulence and antibiotic resistance genes. This background information, presented in chapter 3 discusses the diversity and human health microbiological risks that may be associated with drinking water *Betaproteobacteria*. A major conclusion of this review is the important knowledge gap on the antibiotic resistance and virulence in drinking water *Betaproteobacteria,* in both phenotypic and genetics insights. The review highlights not only the impressive taxonomic diversity but also the occurrence of putative resistance and virulence genetic elements, whose investigation is needed. Members of the genera *Achromobacter*, *Burkholderia*, and *Ralstonia* were interesting candidates for prioritizing such investigation, since besides being common in drinking water, have been associated with infection outbreaks. *Ralstonia*, a genus for which information is scarce, was selected for further studies in this thesis, not only because of their ubiquity in the urban water cycle, but also because of the availability of isolates in the host institution. From a culture collection of *Ralstonia* spp. strains recovered from different aquatic environments it was selected a group of *R. pickettii* and *R. mannitolilytica* strains from wastewater, tap water and mineral water.

A first experimental investigation (Chapter 4,) was based on five strains of *Ralstonia picketti* and *R. mannitolylitica*, which were compared for their antibiotic and metal resistance phenotypes, growth kinetics (growth rate, phase lag and yield) in the presence and absence of antibiotic and metal, biofilm formation and disinfectant (UV radiation, chlorine, and hydrogen peroxide) tolerance. It was observed the coincidence of gentamicin

and arsenite resistance. The hypothesis on the use of common mechanisms of resistance was rejected given the distinct kinetic responses to arsenite and gentamicin. However, both agents enhanced the biofilm formation in resistant strains, suggesting common physiological responses to the metal and antibiotic. The hypothesis that gentamicin resistance might be associated with increased tolerance to disinfectants was proved to hydrogen peroxide, more effective against gentamicin susceptible than resistant strains.

After this first insight, the characterization of the resistance genetic determinants aimed at unveiling the association between arsenite and gentamicin resistance and infering about the stability and potential for horizontal gene transfer of these features. This investigation (Chapter 5) involved 37 strains from mineral water (n=14), tap water (n=17) and hospital wastewater (n=6). The strains were examined for the minimal inhibitory concentration of gentamicin and arsenite, number and size of plasmids and presence of genes related with integrative and conjugative elements (ICEs), arsenite resistance (*arsH* and *acr3*) and a RND efflux system gene (*cmeA*), among other. The analyses of a larger group of strains confirmed the association between gentamicin resistance and increased arsenite tolerance, a property that coincided with the presence of genes of the ICEs and arsenite operon. The separation of the isolates in gentamicin and arsenite resistant *vs.* susceptible isolates was in agreement with the analyses of the genes sequences of 16S rRNA, *cmeA*, $bla_{\text{OXA-22}}$ and $bla_{\text{OXA-60}}$, which consistently separated those isolates into two groups. Given the coherency of the two groups, the whole genome sequences of a resistant and a susceptible isolate were compared for the functional categories "membrane transport", "stress response" and "virulence, disease and defense". The search followed two criteria, the genes only detected in the resistant strain and the genes that had point mutations between both isolates. The results suggested the divergence between both lineages with point mutations in 65 deduced amino acid sequences and 30 genes only detected in the gentamicin and arsenite resistant strain. Moreover, it was suggested that the presence of the ICE in the resistant strains may have facilitated the acquisition of traits such as arsenite resistance.

This thesis was designed to comprise three scientific peer-reviewed publications, Chapter 3 – "*Betaproteobacteria* are predominant in drinking water: are there reasons for concern?", submitted for publication; Chapter 4 – "Association between gentamicin resistance and stress tolerance in water isolates of *Ralstonia pickettii* and *R. mannitolilytica"* (Ferro *et al.,* 2018), and Chapter 5, "Hints for acquired gentamicin and arsenite resistance in *Ralstonia pickettii* water isolates", in preparation.

Chapter 3: ***Betaproteobacteria*** **are predominant in drinking water: are there reasons for concern?**

Submitted for publication:

Authors: Pompeyo Ferro, Ivone Vaz-Moreira and Celia M. Manaia

Title: "*Betaproteobacteria* are predominant in drinking water: are there reasons for concern?"

Journal: Critical Reviews in Microbiology (under review).

Authors contributions:

	Planning	Literature search	Data analyses	Writting
Pompeyo Ferro	x	x	x	x
Ivone Vaz-Moreira	x	x	x	x
Célia M. Manaia	x		x	x

3. *Betaproteobacteria* are predominant in drinking water: are there reasons for concern?

3.1. Abstract

Betaproteobacteria include some of the most abundant and ubiquitous bacterial genera that can be found in drinking water, including mineral water. The combination of physiology and ecology traits place some *Betaproteobacteria* in the list of potential, yet sometimes neglected, opportunistic pathogens that can be transmitted by water or aqueous solutions. Indeed, some drinking water *Betaproteobacteria* with intrinsic and sometimes acquired antibiotic resistance, harboring virulence factors and often found in biofilm structures, can persist after water disinfection and reach the consumer.

This literature review summarizes and discusses the current knowledge about the occurrence and implications of *Betaproteobacteria* in drinking water. Although the sparse knowledge on the ecology and physiology of *Betaproteobacteria* thriving in treated or natural mineral/spring drinking water is evidence of this review, it is demonstrated that drinking water holds a high diversity of *Betaproteobacteria*, whose presence may not be innocuous. Frequently belonging to genera also found in humans, drinking water *Betaproteobacteria* are ubiquitous in different habitats, have the potential to resist antibiotics either due to intrinsic or acquired mechanisms, and hold different virulence factors. The combination of these factors place drinking water *Betaproteobacteria* in the list of candidates of emerging opportunistic pathogens. Improved bacterial identification of clinical isolates associated with opportunistic infections and additional genomic and physiological studies may contribute to elucidate the potential impact of these bacteria.

3.2.Introduction

The access to safe drinking water is defined as one of the Sustainable Development Goals and an important human right (https://www.un.org/sustainabledevelopment/sustainable-development-goals/). By definition, drinking water is suitable for human consumption, washing/showering and domestic food preparation (Bartram *et al.,* 2003; Council directive 98/83/EC, 1998; WHO, 2011). drinking water comprises i) treated water originating from a surface water (river, lagoons, alluvial wells) or groundwater source that, when necessary may be subjected to treatment before distribution to the consumer, and ii) the natural mineral or spring water originating from a groundwater table or deposit that emerges from a spring or borehole exit (Barrell *et al.,* 2000). While the so-called tap-water needs treatment in most world regions, due to the widespread contamination of water sources, the natural mineral or spring water is "microbiologically wholesome" and must not receive any treatment capable of changing the original chemical and microbiological composition (European Comission 2009). Mineral and spring waters are commonly bottled before distribution to the consumer.

The natural mineral and spring waters microbiome comprises the autochthonous bacterial community, although the structure of that bacterial community may change after bottling and storage (Flemming *et al.,* 2016). Otherwise, the treated drinking water microbiome occurring in the water that reaches the consumer does not necessarily mirror that thriving in the water source. This is due to the successive alterations that take place from the source to the tap, due mainly to treatment, reactivation, and piping (Eichler *et al.,* 2006; Hoefel *et al.,* 2005; Lautenschlager *et al.,* 2010; Lautenschlager *et al.,* 2014; Norton and LeChevallier, 2000; Vaz-Moreira *et al.,* 2013). Indeed, the bacterial diversity of treated water results from the selection of the autochthonous bacterial community members that survived the treatment (e.g. chlorination, ozonation or UV irradiation), together with potential intrusions of bacteria throughout the system from the source to the tap. The properties of water and specific physicochemical factors, such as total organic content or hydrodynamic regime, the conditions of the pipes, the range of temperature and pH, the residence time, among others, may influence the shape of the bacterial community (Douterelo

et al., 2013; Lautenschlager *et al.,* 2010, 2014; Pepper *et al.,* 2004; Pinto *et al.,* 2012). Another important driver of the tap water bacterial community composition and structure is the formation of biofilms along the distribution systems, which may rule the release of biofilm bacteria into the circulating water (Batte *et al.,* 2003). Despite the specificities of each water source, piping and treatment conditions, *Proteobacteria* (mainly of the classes Alpha, Beta and Gamma) are among the predominant populations in drinking water, treated or mineral/spring, worldwide (Eichler *et al.,* 2006; Hoefel *et al.,* 2005; Leclerc and Moreau, 2002; Loy *et al.,* 2005; Pinto *et al.,* 2012; Poitelon *et al.,* 2009; Revetta *et al.,* 2010; Vaz-Moreira *et al.,* 2014). Although *Alphaproteobacteria*, and mainly *Gammaproteobacteria*, are many times explored since they include members who are well known pathogenic bacteria, the same is not happening with *Betaproteobacteria* and this was a major motivation to bring forward the current review, focused on *Betaproteobacteria.*

Drinking water is an important source for the dissemination and transmission of microbial agents to humans, meaning that the drinking water microbiome may pose important potential risks for human health. In a previous study, Vaz-Moreira and colleagues (2017) observed that *Proteobacteria* genera can persist after drinking water treatment, being ubiquitous along the drinking water source-treatment-distribution-tap thread. In that study, the ubiquity of *Betaproteobacteria* in the drinking water system was evidenced, confirming previous studies conducted in other clean environments, such as filtered water, antiseptics or disinfectants (Hahn, 2004; Weber *et al.,* 2007). These results are also in line with data reported in studies about bottled natural mineral water, which identify *Betaproteobacteria* among the predominant bacterial groups (França *et al.,* 2015; Leclerc and Moreau, 2002; Loy *et al.,* 2005). The remarkable capacity to form biofilm in freshwater habitats (Araya *et al.,* 2003; Manz *et al.,* 1999) and the survival to disinfectants and disinfection processes (Becerra-Castro *et al.,* 2016; Mi *et al.,* 2015) are probably part of the explanation for the observed ubiquity of *Betaproteobacteria* in drinking water. These evidences claim for the attention of the scientific community mainly because some of the drinking water *Betaproteobacteria* genera may comprise opportunistic pathogens and/or drug resistant bacteria. In this review, we were interested in overviewing what is known about *Betaproteobacteria* ecology, intrinsic or acquired antibiotic resistance and virulence factors, as background information for discussing

potential human health implications and, if justified, identifying relevant knowledge gaps.

3.3.Context and approach

Based mainly on phylogenetic evidence, recently Parks *et al.,* (2018) proposed that the class *Betaproteobacteria* would be reclassified into the order *Betaproteobacteriales,* within the class *Gammaproteobacteria*. For practical reasons, this review followed the NCBI Taxonomy database (https://www.ncbi.nlm.nih.gov/Taxonomy/), in which the class *Betaproteobacteria* comprises 23 families and a large group of unclassified *Betaproteobacteria,* including some groups with Candidatus statute. Most of these 23 families (17) have been reported in drinking water habitats (Figure 3.1). This is not surprising, given the ubiquity of *Betaproteobacteria* that colonizes habitats such as soil and rhizosphere, plants, foods, clinical samples, among other (Garrity *et al.,* 2005), as well as aquatic environments, particularly drinking water (Eichler *et al.,* 2006; Hoefel *et al.,* 2005; Leclerc and Moreau, 2002; Loy *et al.,* 2005; Pinto *et al.,* 2012; Poitelon *et al.,* 2009; Revetta *et al.,* 2010; Vaz-Moreira *et al.,* 2014).

For this review were selected studies that approach the bacterial diversity in water destined for human consumption, both treated water and bottled natural mineral/spring water. This selection included also the bacterial diversity of treated drinking water biofilms, since biofilms are known to strongly influence and result from the tap water bacterial diversity (Berry *et al.,* 2006; Srinivasan *et al.,* 2008). Because human health implications may result from a transient or resident bacterial colonization, we also explored if the *Betaproteobacteria* genera detected in drinking water have been reported in the human microbiome. These analyses were based on the Human Microbiome (https://hmpdacc.org/catalog/) and Human Oral microbiome (http://www.homd.org/) catalogs, and the NCBI database (www.ncbi.nlm.nih.gov) filtering by "Host: Homo sapiens".

From these analyses were listed 24 *Betaproteobacteria* genera that were detected both in treated and bottled natural mineral/spring water and whose association with humans was also reported. Members of these genera were examined for their

potential as carriers/disseminators of virulence or of antimicrobial resistance determinants. The virulence factors were compiled from the literature available and from the Virulence Factors Database (VFDB, http://www.mgc.ac.cn/VFs/). Intrinsic and acquired antimicrobial resistance was compiled from the literature available.

3.4. *Betaproteobacteria* in drinking water

As mentioned above a total of 17 *Betaproteobacteria* families, belonging to six orders, were reported in drinking water habitats. The most commonly reported families (*Comamonadaceae, Oxalobacteraceae, Burkholderiaceae, Alcaligenaceae* and unclassified *Burkholderiales*), represented by 54 out of 83 genera, belong to the order *Burkholderiales* (Figure 3.1). A total of 63 bacterial genera were identified in natural mineral/spring water and 55 in treated drinking water. Among those, 36 genera were reported in both mineral/spring and treated drinking water. These bacteria were members of 5 of the 6 orders of *Betaproteobacteria* described in drinking water: *Burkholderiales* (25 genera), *Rhodocyclales* (5 genera), *Neisseriales* (2 genera), *Nitrosomonadales* (2 genera), *Hydrogenophilales* (1 genus), and *Methylophilale*s (1 genus) (Figure 3.1). This distribution suggests the endemic character of bacteria of these orders to drinking water, independently of being treated or mineral/spring. In contrast, some *Betaproteobacteria* were only reported in natural mineral/spring water habitats, and, to our knowledge, were never reported in treated drinking water water (e.g. *Pseudorhodoferax*, *Brachymonas, Ottowia, Caenimonas, Alicycliphilus, Ramlibacter, Diaphorobacter, Xenophilus, Xylophilus, Leptothrix, Piscinibacter, Tepidimonas, Oxalobacter, Telluria, Paucimonas, Derxia, Alcaligenes, Methylobacillus, Sulfuritalea, Azoarcus, Deefgea,* and *Ferritrophicum*) (Figure 3.1). This may suggest the influence of physiologic and metabolic properties of these bacteria and/or their susceptibility to water treatment.

As expected, most of the bacterial genera observed in treated drinking water biofilms were also observed in the bulk treated drinking water (27 out of 33 genera), being the exception the genera *Sutterella, Undibacterium, Neisseria, Methylibium, Methylotenera*, and *Methylovorus*. Most of the genera observed to be ubiquitous in drinking water were also reported in association with humans (24 out of the 36:

Achromobacter, Ralstonia, Limnobacter, Burkholderia, Cupriavidus, Acidovorax, Delftia, Polaromonas, Curvibacter, Variovorax, Comamonas, Pelomonas, Malikia, Herminiimonas, Janthinobacterium, Herbaspirillum, Massilia, Aquabacterium, Ideonella, Chromobacterium, Methylophilus, Dechloromonas, Propionivibrio, and *Azospira*) (Figure 3.1). Members of these genera represent candidates able to establish an association with the human microbiome, leading to the eventual transfer of resistance to antibiotics, or posing other risks to human health.

Not much is known about the influence of drinking water bacteria in the human gut and in what conditions drinking water bacteria can represent a risk for human health. The importance of drinking water as a vehicle of *Betaproteobacteria* was highlighted by Lee *et al.,* (2010), who used germ-free mice to demonstrate a correlation between the bacterial communities originating in the drinking water and those present in the gastrointestinal tract, with the *Betaproteobacteria Ralstonia* representing one of the bacterial genera transported to the gastrointestinal tract via drinking water. Recently, Dias *et al.,* (2018) studied the response of the mouse gut bacterial community to the ingestion of different types of drinking water. After 23 days of water consumption it was observed a significant increase in feces of the relative abundance of *Firmicutes* for the different types of water, and of *Acinetobacter* and *Staphylococcus* spp. for treated tap water.

3.4.1. Survival strategies

Betaproteobacteria comprise bacteria with the capacity to survive disinfectants or disinfection processes (Garrity *et al.,* 2005; Mi *et al.,* 2015; Williams *et al.,* 2004), which facilitate the persistence of these bacteria in drinking water treatment systems. These properties may explain the *Betaproteobacteria* dominance in chloraminated water (Williams *et al.,* 2004), and their high fitness in the presence of elevated chlorine dosage, becoming the largest *proteobacterial* class in the drinking water distribution system (Mi *et al.,* 2015). The resilience of drinking water *Betaproteobacteria* is also demonstrated in different reports where they are described as contaminants of sterile solutions or of disinfectant solutions (Weber *et al.,* 2007). For example, *Ralstonia* spp. are often reported as contaminants in blood culture

medium, sterile saline solution or other medical solutions (Boutros *et al.,* 2002; Gardner and Shulman, 1984; Grobner *et al.,*, 2007; Labarca *et al.,* 1999; Lacey and Want, 1991; Luk, 1996; Maki *et al.,* 1991; Maroye *et al.,* 2000; McNeil *et al.,* 1985; Roberts *et al.,* 1990). Also, *Burkholderia* spp. (Doit *et al.,* 2004; Estivariz *et al.,* 2004; Held *et al.,* 2015; Ko *et al.,* 2015; Magalhaes *et al.,* 2003; Nasser *et al.,* 2004), and *Achromobacter* spp. (Hugon *et al.,* 2015; Tena *et al.,* 2005; Turgutalp *et al.,* 2012; Vu-Thien *et al.,* 1998) have been reported as contaminations of disinfectants solutions and medications. This capacity to survive disinfectants or disinfection processes may explain the high diversity of *Betaproteobacteria* observed in treated drinking water (Figure 3.1).

Associated with the capacity to survive treatment processes (e.g. disinfectants, toxic metals, antibiotics), the capacity of *Betaproteobacteria* to form biofilms is frequently described (Emtiazi *et al.,* 2004; Ferro *et al.,* 2018; Flemming *et al.,* 2016; Mah and O'Toole, 2001; Schwering *et al.,* 2013). The association between both characteristics may have two explanations: i) the bacteria with increased fitness to survive antimicrobial agents are those able to form or incorporate biofilm structures, or ii) the biofilm provides an increased protection against external attacks (e.g. disinfectants) working as a kind of shield by inhibiting the antimicrobial diffusion by the extracellular polymeric substance (EPS) molecules or by a direct consequence of the slow growth state of the biofilm cells avoiding drugs that target metabolic processes occurring during growth (Anderson and O'Toole 2008; Berry *et al.,* 2006; Dufour *et al.,* 2012; Flemming *et al.,* 2016; Lewis, 2001; Schwering *et al.,* 2013). Indeed, both mechanisms are probably combined, as is reported for example for *Ralstonia pickettii*, able to survive disinfectant solutions and form biofilm in industrial and pharmaceutical high-purity water systems (Adley *et al.,* 2005; Kulakov *et al.,* 2002; Ryan *et al.,* 2011). In drinking water, it was observed that most of the bacterial genera reported in biofilms were also reported in treated drinking water (e.g. *Ralstonia, Limnobacter, Burkholderia, Cupriavidus, Acidovorax, Delftia, Polaromonas, Curvibacter, Variovorax, Janthinobacterium, Herbaspirillum, Aquabacterium, Dechloromonas*), suggesting that these bacteria exist in a dynamic equilibrium between the planktonic and biofilm state. However, some genera, described mainly in biofilms rather than in the planktonic state in drinking water, such as *Sutterella, Undibacterium, Neisseria, Methylibium,*

Methylotenera, and *Methylovorus,* may benefit from the protective biofilm structure (Figure 3.1).

Other mechanisms, such as the association with free-living amoebas, may also explain the good fitness of the *Betaproteobacteria* in drinking water. The free-living amoebas can easily resist the drinking water treatment and are important in the bacterial community modulation since they feed on bacteria, by phagocytosis (Delafont *et al.,* 2016). However, some bacteria developed mechanisms of amoeba-digestion resistance, and instead of dying when internalized by amoeba, they survive and multiply, being later released back to the environment. Some *Betaproteobacteria* comprise amoeba-resistant members, as has been described for the genera *Achromobacter, Burkholderia, Chromobacterium, Delftia,* and *Ralstonia* (Thomas *et al.,* 2010). Curiously, all of these genera have been reported in both treated and mineral drinking water as well as in the human microbiome (Figure 3.1).

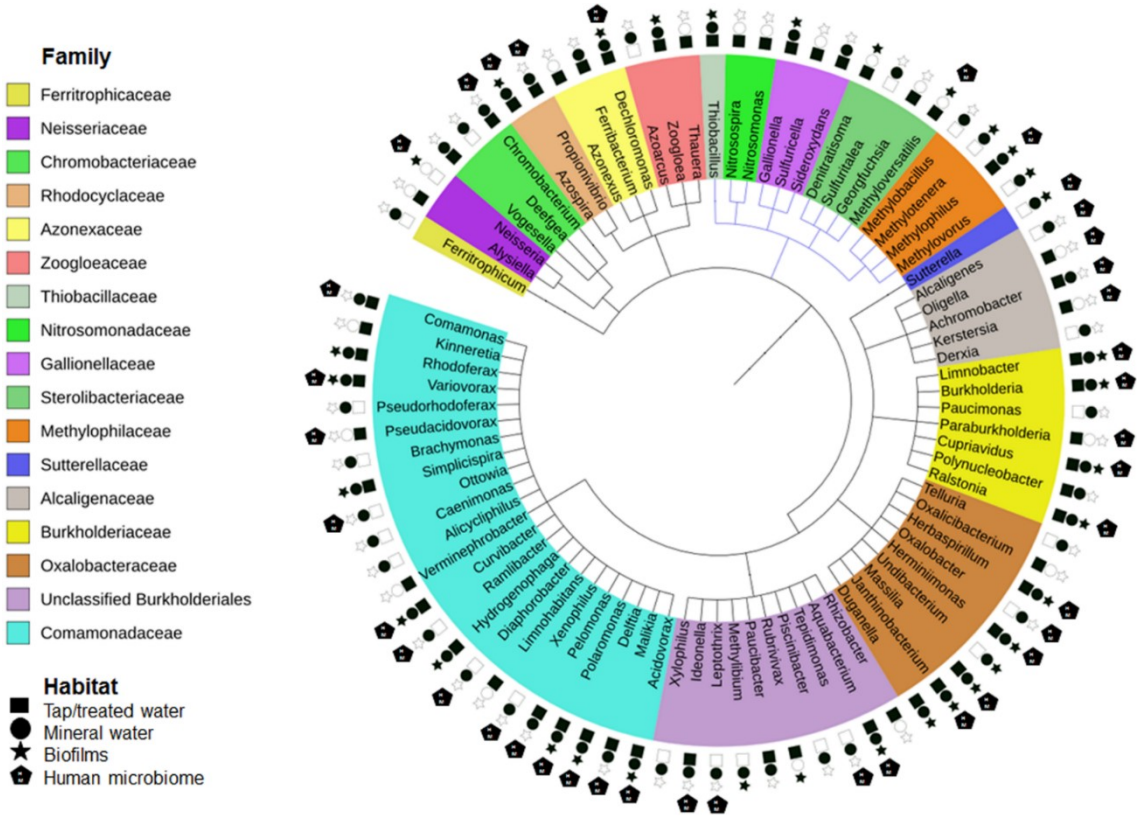


Figure 3.1. Diversity of *Betaproteobacteria* in drinking water habitats and in the Human microbiome.

The black symbol means "detected", the white "non-detected". The dendrogram was constructed with the iTOL – interactive tree of life (Letunic and Bork, 2016), based on the taxon ID codes.

3.5. Drinking water *Betaproteobacteria* as potential carriers of virulence factors

Virulence factors are molecules that enable a microorganism to establish itself on or within a host and enhance its potential to cause disease. The virulence of a pathogen depends on its ability to accomplish the different steps required to cause an infection: adhesion, colonization, invasion, immune response inhibition and/or production of toxins. Therefore, the most complex is the virulence machinery, the most successful a pathogen is. With modest virulence machinery, opportunistic pathogens are commensal or environmental bacteria, often innocuous for a healthy individual. However, these bacteria, have the potential to cause disease in individuals with diminished defenses (e.g., disease, wound, medication, prior infection, immunodeficiency, ageing), due to the presence of virulence factors that facilitate invasion and or proliferation in the host (Brown *et al.,* 2012). Some of the *Betaproteobacteria* found in drinking water have a distinct array of virulence factors and, therefore, meet the criterion of opportunistic pathogens (Table 3.1).

Virulence factors or homologous genes have been described in 11 out of the 24 *Betaproteobacteria* genera detected in both drinking water (treated and mineral) and in the human microbiome (Table 3.1). The fact that only these 11 genera were reported as potential carriers of virulence factors suggests a major knowledge gap about ubiquitous and potentially hazardous microbial groups. Curiously, not even for species associated with outbreaks, as *Ralstonia pickettii* and *R. mannitolilytica*, were described virulence factors (Coman *et al.,* 2017; Daxboeck *et al.,* 2005; Grobner *et al.,* 2007; Labarca *et al.,* 1999; Maroye *et al.,* 2000).

Virulence factors may be divided into membrane proteins, capsule, secretory proteins, and others (Table 3.1). The membrane proteins are mainly associated with the increased capacity of adhesion of the bacteria to the host cells (Wu *et al.,* 2008). Specifically, the presence of type IV pili, a mechanism restricted to Gram-negative bacteria and also one of the most common, not-surprisingly was the most common among the drinking water *Betaproteobacteria* (Table 3.1). The presence of a capsule, a key virulence determinant that can mediate resistance to both phagocytosis and complement-mediated killing (Abreu and Barbosa, 2017; Reckseidler-zenteno *et al.,* 2005), was described in *Burkholderia* species. The secretory proteins include the systems of transport of toxins, the toxins, and immune response inhibitors, as well as

other siderophores or proteins, all of them observed in drinking water *Betaproteobacteria* (Table 3.1). Secretion systems are used by bacteria to secrete virulence factors from the cytosol into host cells or the host environment, and can span both the inner and outer membrane (e.g. RND efflux systems, T1SS, T2SS, T3SS, T4SS, T6SS) or just the outer membrane (e.g. T5SS) (Costa *et al.,* 2015). In human-associated drinking water *Betaproteobacteria*, the most common secretion systems seem to be T2SS, T3SS, and T6SS (Table 1). One of those, the T3SS, also known as "injectisome", has an important role in the proteins export from the bacterial cytoplasm into the host eukaryotic cells (Cornelis, 2006; Puhar and Sansonetti, 2014), being the mechanism used *by B. pseudomallei* to cause melioidosis in mammals or *R. solanacearum* to cause plant bacterial wilt (Puhar and Sansonetti, 2014; Stevens *et al.,* 2002; Valls *et al.,* 2006). The multidrug RND (resistance nodulation cell division) efflux pumps, described for *B. pseudomallei* (Table 3.1), may be responsible for intrinsic resistance to several antimicrobials (Munita and Arias, 2016; Rhodes and Schweizer, 2016). T4SS, only described for *B. cenocepacia* and *A. xylosoxidans* (Table 3.1), allow the transport of DNA and may have an important role in the transfer of genetic material (Cascales and Christie, 2013; Green and Mecsas, 2016). Toxin production is described in members of the genera *Burkholderia, Chromobacterium*, and *Achromobacter* (Table 3.1).

Quorum-sensing (QS) is a bacterial cell-to-cell communication process, based on auto-inducer signaling, enabling bacteria to adjust the cell density and gene expression, regulating activities such as bioluminescence, sporulation, competence, antibiotic production, biofilm formation, or virulence factor secretion (Rutherford and Bassler, 2012). QS is important in biofilm formation and also for the activation of virulence factors (Dufour *et al.,* 2012; Soto, 2013). These communication processes have been described in some *Burkholderia* spp. and *Chromobacterium violaceum, Ralstonia solanacearum*, or *Polaromonas* spp. (Table 3.1).

This review on virulence factors reveal that the machinery for host colonization, invasion and infection, typical of opportunistic pathogens, is available in drinking water *Betaproteobacteria* that can also be associated with the human microbiome. Potential virulence may be not eliminated by disinfection as was demonstrated by previous studies that demonstrated that chlorination may promote an increase of the relative abundance of virulence proteins in drinking water (e.g. translocases,

transposons, Clp proteases, and flagellar motor switch proteins) . Potential virulence combined with disinfection resilience put drinking water *Betaproteobacteria* among the potential relevant safety biomarkers.

Table 3.1 Described virulence factors or homologous genes (*) in *Betaproteobacteria* genera observed in treated and mineral drinking water and described as human-associated bacteria

Classification	Sub-classification	Examples	Drinking-water associated bacteria	References
Membrane proteins	Adhesion	*Burkholderia* oligomeric coiled-coil adhesin A (*BoaA*) and b (*BoaB*).	*Burkholderia pseudomallei*	(Balder *et al.,* 2010)
		Pilus structural proteins (Type IV pili)	*B. pseudomallei*; *Burkholderia cenocepacia*; *Acidovorax avenae subsp. avenae*; *Acidovorax citrulli*; *Ralstonia solanacearum*; *Limnobacter thiooxidans* (*); *Chromobacterium violaceum* (*)	(Alves De Brito *et al.,* 2004; Andrews, 2001; Bahar *et al.,* 2009; Burdman and Walcott, 2012; Essex-lopresti *et al.,* 2005; Har *et al.,* 2015; Holden *et al.,* 2009; Ibrahim *et al.,* 2012; Kang *et al.,* 2002; Liu *et al.,* 2001; Stone *et al.,* 2014)
		Chaperone-usher type fimbriae	*B. cenocepacia*	(Holden *et al.,* 2009)
		Flp-type pili	*B. cenocepacia*; *Cupriavidus taiwanensis* (*)	(Amadou *et al.,* 2008; Holden *et al.,* 2009)
		Hemagglutinin/hemolysin related	*B. pseudomallei* (*); *L. thiooxidans* (*); *Achromobacter xylosoxidans* (*)	(Dowling *et al.,* 2010; Har *et al.,* 2015; Li, *et al.,* 2013)
		Mannose-fucose binding lectin (LecM)	*R. solanacearum*	(Meng *et al.,* 2015)
		22-Kda adhesion protein AdhA	*B. cenocepacia*	(Holden *et al.,* 2009)
		BuHA family of proteins	*B. cenocepacia*	(Holden *et al.,* 2009)
		BcaA autotransporter protein	*B. pseudomallei*	(Campos *et al.,* 2013; Stone *et al.,* 2014)
		poly-β-1,6-N-acetyl-D-glucosamin (*pga* operon)	*A. xylosoxidans* (*)	(Jakobsen *et al.,* 2013)
		Outer Membrane Protein (Omp21)	*Delftia acidovorans*	(Baldermann *et al.,* 1998)
	Actin-based intracellular motility	*Burkholderia* intracellular motility A (BimA)	*B. pseudomallei, Burkholderia mallei; Burkholderia thailandensis*	(Sitthidet *et al.,* 2011, 2010; Stevens *et al.,* 2005)
	Invasion and colonization	Polar flagella	*B. pseudomallei*; *B. cenocepacia*; *A. citrulli*	(Burdman and Walcott, 2012; Chua *et al.,* 2003; Inglis *et al.,* 2003; Urban *et al.,* 2004)

		BuHA family of autotransporting membrane proteins	*B. cenocepacia*	(Holden *et al.,* 2009)
	Surface components	LPS core oligosaccharide	*B. cenocepacia*; *A. xylosoxidans* (*); *C. violaceum* (*)	(Alves De Brito *et al.,* 2004; Genin and Denny, 2012; X. Li *et al.,* 2013; Loutet and Valvano, 2010)
		EPS (extracellular polysaccharide)	*R. solanacearum*	
	Others	HtrA protease	*B. cenocepacia*	(Colburn-Clifford and Allen, 2010; Flannagan *et al.,* 2007)
		cbb3-Type Cytochrome c Oxidase	*R. solanacearum*	
Capsule	Antiphagocytosis	Type I O-polysaccaharide (capsule I)	*B. pseudomallei*	(DeShazer *et al.,* 1998; Reckseidler-zenteno *et al.,* 2005; Wikraiphat *et al.,* 2009)
		Cepacian polysaccharide	*B. cenocepacia*	(Holden *et al.,* 2009)
		Capsular polysaccharides (CPS)	*B. pseudomallei, B. thailandensis*	(Cuccui *et al.,* 2012; Marchetti *et al.,* 2015; Reckseidler-zenteno *et al.,* 2005)
Secretory proteins	Immune response inhibitors	Mip-like (macrophage infectivity potentiator)	*C. taiwanensis* (*)	(Amadou *et al.,* 2008)
		Proteases	*B. pseudomallei* (*)	(Dowling *et al.,* 2010)
		Phospholipases	*B. pseudomallei* (*)	(Dowling *et al.,* 2010)
		TssM (BPSS1512) deubiquitinase	*B. pseudomallei*	(Tan *et al.,* 2010)
	Toxins	HicA toxin	*B. pseudomallei*	(Butt *et al.,* 2014)
		Bcc toxin	*Burkholderia cepacia* complex	(Thomson and Dennis, 2012)
		Burkholderia Lethal Factor 1 (BLF1)	*B. pseudomallei*	(Cruz-Migoni *et al.,* 2011)
		Hemolysin	*B. cepacia*; *B. pseudomallei* (*); *C. violaceum* (*)	(Alves De Brito *et al.,* 2004; Dowling *et al.,* 2010; Hutchison *et al.,* 1998)
		RTX toxin	*A. xylosoxidans* (*)	(Li *et al.,* 2013)
		Colicin V and exoenzyme regulatory protein (AepA)	*A. xylosoxidans* (*); *C. violaceum* (*)	(Alves De Brito *et al.,* 2004; Jakobsen *et al.,* 2013)
	Transport of toxins	RND efflux pump (e.g. BpeAB-OprB)	*B. pseudomallei*	(Chan and Chua, 2005; Mima and Schweizer, 2010)

	Type I secretion system (T1SS)	*B. pseudomallei*; *B. cenocepacia*; *C. violaceum* (*)	(Alves De Brito *et al.,* 2004; Holden *et al.,* 2009, 2004)
	Type II secretion system (T2SS)	*B. pseudomallei*; *B. mallei*; *B. cenocepacia; R. solanacearum*; *A. avenae subsp. avenae* (*)*; A. citrulli* (*)*; C. taiwanensis* (*)*; L. thiooxidans* (*); *C. violaceum* (*); *A. xylosoxidans* (*)	(Amadou *et al.,* 2008; Burdman and Walcott, 2012; Har *et al.,* 2015; Holden *et al.,* 2004, 2009; Ibrahim *et al.,* 2012; Persson *et al.,* 2009; Poueymiro and Genin, 2009)
	Type III secretion system (e.g. Bsa T3SS)	*B. pseudomallei*; *B. mallei*; *B. thailandensis*; *B. cenocepacia; R. solanacearum*; *A. citrulli; Herbaspirillum rubrisubalbicans*; *A. avenae subsp. avenae* (*); *C. taiwanensis* (*); *Limnobacter sp.* (*); *C. violaceum* (*); *A. xylosoxidans* (*)	(Alves De Brito *et al.,* 2004; Amadou *et al.,* 2008; Cullinane *et al.,* 2008; Genin *et al.,* 2005; Holden *et al.,* 2004, 2009; Ibrahim *et al.,* 2012; Jakobsen *et al.,* 2013; Kondo *et al.,* 2017; Li *et al.,* 2013; Muangman *et al.,* 2011; Poueymiro and Genin, 2009; Schmidt *et al.,* 2012; Stevens *et al.,* 2003)
	Type IV secretion system (T4SS)	*B. cenocepacia*; *A. xylosoxidans* (*)	(Engledow *et al.,* 2004; Li *et al.,* 2013)
	Type V secretion system (T5SS)	*B. pseudomallei*; *B. mallei*; *B. cenocepacia*; *Limnobacter* sp. (*)	(Holden *et al.,* 2004, 2009; Persson *et al.,* 2009)
	Type VI secretion system (e.g. T6SS-5)	*B. pseudomallei*; *B. mallei*; *B. cenocepacia*; *B. thailandensis*; *A. avenae subsp. avenae*; *A. citrulli*; *C. taiwanensis* (*); *L. thiooxidans* (*); *Limnobacter* sp. (*); *A. xylosoxidans* (*)	(Amadou *et al.,* 2008; Har *et al.,* 2015; Holden *et al.,* 2009; Ibrahim *et al.,* 2012; Jakobsen *et al.,* 2013; Persson *et al.,* 2009; Schell *et al.,* 2008; Schwarz *et al.,* 2010; Tian *et al.,* 2015)
Other	Zinc metalloproteases ZmpA and ZmpB	*B. cenocepacia*	(Holden *et al.,* 2009)
	Phospholipases C	*B. cenocepacia*	(Holden *et al.,* 2009)
	Siderophores (e.g. ornibactin, salicylic acid, pyochelin, staphyloferrin B, micacocidin)	*B. cenocepacia; R. solanacearum*; *L. thiooxidans* (*)	(Bhatt and Denny, 2004; Har *et al.,* 2015; Holden *et al.,* 2009; Kreutzer *et al.,* 2011; Sokol *et al.,* 1999)
	bipB, bipC and bipD proteins	*B. pseudomallei*	(Stone *et al.,* 2014; Vander Broek and Stevens, 2017)

		Malleipeptin A and malleipeptin B	*B. pseudomallei*	(Biggins *et al.,* 2014)
		MprA serine metalloprotease	*B. pseudomallei*	(Burtnick *et al.,* 2014; Valade *et al.,* 2004)
		MgtC protein	*B. cenocepacia*	(Rang *et al.,* 2007)
Others	Biofilm production	FixLJ system	*B. cepacia* complex	(Schaefers *et al.,* 2017)
		Lys-R type regulator	*B. cenocepacia*; *R. solanacearum*	(Bernier *et al.,* 2008; Brumbley *et al.,* 1993; Schell, 2000)
		Mannose-fucose binding lectin (LecM)	*R. solanacearum*	(Meng *et al.,* 2015)
	Phenylacetic acid catabolic pathway		*B. cenocepacia*	(Law *et al.,* 2008)
	Denitrification	Nitrate reduction (e.g. Nos system, NirV)	*A. xylosoxidans* (*)	(Jakobsen *et al.,* 2013)
	Signalling	c-di-GMP-specific phosphodiesterase (CdpA)	*B. pseudomallei*	(Lee *et al.,* 2010)
		CepIR Quorum-sensing system	most *Burkholderia* spp.	(Chan and Chua, 2005; Holden *et al.,* 2009; Lewenza *et al.,* 1999; Song et al., 2005; Subramoni and Sokol, 2012; Subsin *et al.,* 2007; Ulrich *et al.,* 2004)
		CciIR Quorum-sensing system	*B. cenocepacia*	(Baldwin *et al.,* 2004)
		BDSF, nonhomoserine lactone signal molecule	*B. cenocepacia*	(Boon *et al.,* 2008)
		BviIR Quorum-sensing system	*B. vietnamiensis*	(Malott and Sokol, 2007)
		PmlI-PmlR Quorum-Sensing System	*B. pseudomallei*	(Valade *et al.,* 2004)
		Violacein (CviI/R AHL QS system)	*C. violaceum*	(Steindler and Venturi, 2007)
		other Quorum sensing systems	*A. citrulli; R. solanacearum*; *Polaromonas* spp. (*)	(Johnson and Walcott, 2013; Meng *et al.,* 2015; Spirig *et al.,* 2008; Wang *et al.,* 2016)

3.6. Antimicrobial resistance in drinking water *Betaproteobacteria*

In addition to the ubiquitous character and virulence potential, some *Betaproteobacteria* exhibit resistance to different antibiotics (Khan *et al.,* 2016; Vaz-moreira *et al.,* 2017; Vaz-Moreira *et al.,* 2014), which may increase the risk associated with their presence in drinking water. Jia *et al.,* (2015) demonstrated that the relative abundance of antibiotic resistance genes (ARGs) increased after drinking water chlorination, being *Betaproteobacteria Acidovorax* spp. among the bacterial groups that most contributed to that shift. Also in natural mineral/spring water, not subjected to any kind of treatment, the presence of *Betaproteobacteria* yielding antibiotic resistance phenotypes has been reported (Falcone-Dias *et al.,* 2012; Messi *et al.,* 2005). These evidences suggest the important contribution of *Betaproteobacteria* to the drinking water resistome.

Although most of the antimicrobial resistance mechanisms detected in the environment can be intrinsic, meaning they are a phenotypic expression of a gene that is common to all members of a given species or genus, they can still contribute to the failure of antibiotic therapy (Cox and Wright, 2013; Perry *et al.,* 2014). A well-known example of intrinsic resistance is the presence of the outer membrane (OM) in Gram-negative bacteria that may modify their porin channels to confer impermeability to different molecules or the presence of efflux pumps that allow the reduction of the intracellular concentration of a given drug contributing to multidrug resistance (MDR) phenotype (Cox and Wright, 2013; Perry *et al.,* 2014; Pothula *et al.,* 2016). The intrinsic resistance is inherited vertically, from one generation to the next.

Different intrinsic antimicrobial resistance mechanisms are described in *Betaproteobacteria* species, although this information is available for a reduced number of species, specifically for six out of the 36 genera reported in both treated and natural mineral/spring drinking water (Table 3.2). This information scarcity is also related with the limited attention that has been given to this group of bacteria, with the exception of a few species that are considered of high clinical relevance (e.g. *Achromobacter xylosoxidans* and *Burkholderia cepacia*). The drinking water *Betaproteobacteria* intrinsic resistance is frequently against penicillins and cephalosporins, as well as to other antimicrobial agents, as fosfomycin (Table 3.2).

It is important to note that some of the species related to the bacterial genera commonly found in drinking water habitats present intrinsic resistance to some drugs that are considered last-resort drugs, being only used in clinical settings. For example, the colistin (polymyxin E) is the only clinically approved therapeutic agent that inhibits the OM and efflux systems (Cox and Wright, 2013). However, some *Burkholderia* spp., *Chromobacterium violaceum* and *Janthinobacterium lividum* are described as being intrinsically resistant to colistin (Table 4.2), and are also reported as infectious agents (Hu and Wang 2012; Jones *et al.,* 2001; Kennedy *et al.,* 2007; Patijanasoontorn *et al.,* 1992; Sirinavin *et al.,* 2005; Yang and Li, 2011; Yuan *et al.,* 2006). Also beta-lactams are frequently used as front-line treatments in combination antibiotic/beta-lactamase inhibitor (e.g. sulbactam, clavulanate, tazobactam) (Cox and Wright, 2013). However, also to these combinations were detected intrinsic resistance phenotypes for *Achromobacter xylosoxidans* and *Burkholderia cepacia* (Table 3.2). Aminoglycosides resistance, described in *Burkholderia* spp. or *A. xylosoxidans* (Table 3.2), is supposedly intrinsic and may be associated to the presence of RND multidrug efflux pumps (e.g. BpeAB-OprB, AmrAB-OprA or AxyXY-OprZ) (Bador *et al.,* 2013; Buroni *et al.,* 2009). This is particularly relevant when some studies show that the occurrence of the RND efflux systems increases in drinking water after chlorination (Jia *et al.,* 2015). The association of these efflux systems to an increased tolerance or resistance to aminoglycosides is quite curious because previous studies have shown a higher prevalence of resistance to aminoglycosides after drinking water treatment (Armstrong *et al.,* 1982; Ma *et al.,* 2017; Narciso-Da-Rocha *et al.,* 2013; Vaz-Moreira *et al.,* 2011, 2012). Although intrinsic resistance has a low potential to be transferred to other bacteria, it may jeopardize the treatment of infections caused by these bacteria.

In addition, some of the described *Betaproteobacteria* characteristics may contribute to their capacity to acquire new resistance to antibiotics, as the capacity to form biofilms and the presence of type 4 secretory systems (T4SS) (Table 3.1). While the T4SS allows the transport of DNA, the biofilm formation allows a close proximity between cells, facilitating both the dissemination of resistance genes between cells by horizontal gene transfer (HGT) (Cascales and Christie, 2013; Flemming *et al.,* 2016; Green and Mecsas, 2016). Krol *et al.,* (2013) observed that conjugation can be up to 700 fold more efficient in biofilms than in free-living bacterial cells.

Described examples are the *A. xylosoxidans* acquired resistance to ciprofloxacin, ceftazidime and carbapenems, in clinical isolates from cystic fibrosis patients (Amoureux *et al.,* 2013) and the acquisition of new genetic elements associated to mobile genetic elements (El Salabi *et al.,* 2012; Hu *et al.,* 2014; Iyobe *et al.,* 2016; Neuwirth *et al.,* 2006; Riccio *et al.,* 2001; Seob *et al.,* 2005; Yamamoto *et al.,* 2012), or the *Burkholderia* spp. acquired antibiotic resistance to fluoroquinolones, trimethoprim among others (Pitt *et al.,* 1996; Rhodes and Schweizer, 2016; Thibault *et al.,* 2004). Apart from these two genera, no information is available for possible acquired antibiotic resistance. Of special interest in this bacterial group, can be the occurrence of co-resistance or cross-resistance. Co-resistance facilitates that the selection of resistance to one agent (e.g. antibiotics, metals, disinfectants) leads to the selection of resistance to the other. Cross-resistance occurs when the same mechanism of resistance is used to avoid different antimicrobial agents (e.g. MDR efflux pumps), conferring the simultaneous resistance to different antimicrobial agents (Baker-Austin *et al.,* 2006; Chapman, 2003).

Table 3.2 Described intrinsic antimicrobial resistance in *Betaproteobacteria* species belonging to bacterial genera detected in both treated and natural mineral/spring drinking water

Species	Beta-lactams				Aminogly-cosides	Polype-ptides	Quinolones	Sulfonamides	Tetracyclines	Others	References
	Penicillins	Cephalosporins	Carbapenems	Monobactam							
Achromobacter xylosoxidans	Ampicillin, Amoxicillin-clavulanate,	Cefazolin, Cefotaxime, Ceftriaxone, Cefepime	Ertapenem	Aztreonam	+	n.i.	n.i.	n.i.	n.i.	Trimethoprim, Fosfomycin	(Abbott and Peleg, 2015; Almuzara *et al.,* 2010; Bador *et al.,* 2013; Leclercq *et al.,* 2013)
Burkholderia cepacia	Ampicillin, Amoxicillin, Piperacillin, Ticarcillin, Ampicillin-sulbactam, Amoxicillin-clavulanate, Piperacillin-tazobactam, Ticarcillin-clavulanate	Cefotaxime, Ceftriaxone, Ceftazidime, Cefepime, Cefsulodin Cefazolin.	Imipenem, Meropenem, Ertapenem	Aztreonam	+	Colistin	Ciprofloxacin	Trimethoprim-sulfamethoxazole	Tetracyclines	Tigecycline, Trimethoprim, Fosfomycin, Chloramphenicol	(Abbott and Peleg, 2015; Baxter et al.,1997; CLSI, 2015; Leclercq *et al.,* 2013; Palleroni, 2005)
Burkholderia gladioli	Ticarcillin, Ticarcillin-clavulanate	Cefsulodin	Imipenem	n.i.	+	Colistin	n.i.	n.i.	n.i.	Fosfomycin	(Baxter *et al.,* 1997; Palleroni, 2005)
Burkholderia mallei	Ticarcillin	n.i.	n.i.	n.i.	n.i.	n.i.	Norfloxacin	n.i.	n.i.	Fosfomycin, Clindamycin	(Thibault *et al.,* 2004)
Burkholderia pseudomallei	Ticarcillin	Cefoxitin	n.i.	n.i.	Gentamicin, Streptomycin, Erythromycin	n.i.	Norfloxacin	n.i.	n.i.	Fosfomycin, Clindamycin	(Buroni *et al.,* 2009; Thibault *et al.,* 2004)
Chromobacterium violaceum	Penicillin, Ampicillin	Cephaloridine	n.i.	n.i.	n.i.	Colistin	n.i.	Sulfafurazole	n.i.	Vibriostatic agent O/129	(Gillis and Logan, 2005a)
Herbaspirillum seropedicae and H. rubrisubalbicans	Pennicilin	n.i.	n.i.	n.i.	n.i.	n.i.	Nalidixic acid	n.i.	n.i.	Novobiocin, Rifampicin	(Baldani *et al.,* 2005)

Janthinobacterium agaricidamnosum	Pennicilin	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	Vancomycin	(Gillis and Logan, 2005b; Lincoln et al., 1999)
Janthinobacterium lividum	Pennicilin	n.i.	n.i.	n.i.	n.i.	Colistin	n.i.	n.i.	n.i.	Nitrofurantoin, Vibriostatic agent O/129	(Gillis and Logan, 2005b)
Variovorax paradoxus	Ampicillin, Methicillin	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	Novobiocin	(Willens *et al.,* 2005)

+, described intrinsic resistance; n.i., no information available.

3.7. Concluding remarks and future research challenges

Water quality is a central issue for human health and wellbeing. On average, an adult ingests about 1 L of water per day, every day. This makes of water the food product ingested at the highest amounts during a person lifetime. Simultaneously, water is also an important way of dissemination of bacteria and chemical compounds, including contaminants (WHO, 2012). For these reasons, drinking water microbiome may play an important role in human health and wellbeing, with relevant implications of the major populations, such as *Betaproteobacteria*. While some drinking water bacteria may be beneficial or innocuous, others may represent a risk for human health. The latter may be due to some drinking water *Betaproteobacteria.*

Betaproteobacteria are abundant and diverse in drinking water or drinking water biofilms, being some of them ubiquitous to treated drinking water and natural mineral/spring water (Figure 3.1). Moreover, some drinking water *Betaproteobacteria* are also reported in humans. The human health risk posed by drinking water *Betaproteobacteria* can be inferred from their resistance to disinfection, the presence of virulence factors and intrinsic antibiotic resistance. All these are factors that may increase the probability of causing opportunistic infections. Some of the virulence factors described in *Betaproteobacteria*, such as adherence factors or the capacity to form biofilms, may contribute to explain the ability of these bacteria to survive in water habitats.

From the literature review, three bacteria genera seem to stood out: *Achromobacter, Burkholderia*, and *Ralstonia*. Members of these genera were also those previously associated with infection outbreaks. Given the phylogenetic and physiologic proximity, other *Betaproteobacteria* genera might share similar properties still unknown, given the scarcity of information. This was, indeed, a major conclusion of this review. Bacteria that are not considered primary pathogens are, most of the times, not screened in routine monitoring analyses in clinical situations. For example, *Ralstonia* spp. have been reported as a nosocomial infectious agent. However, is rarely associated with infection episodes, probably due to misidentifications of these bacteria (Coman *et al.,* 2017; Daxboeck *et al.,* 2005; Ryan and Adley, 2014; Ryan *et al.,* 2006).

The first step to improve the current knowledge is to have a good overview of the *Betaproteobacteria* diversity in drinking water and their possible association with humans, virulence, adaption potential, and genome dynamics for antimicrobial resistance or virulence acquisition. This review is a first step to fill in this gap. Because some of those characteristics are only possible to study having a bacterial culture, it is important to invest more in the development of culturomic approaches (Greub, 2012; Lagier *et al.,* 2012) to obtain drinking water isolates.

Although drinking water is considered important for human health and well-being, many questions are still requiring our attention. It is important to understand how/if the drinking water microbiota, including the *Betaproteobacteria* group, focused in this review, may direct or indirectly influence the human health.

Chapter 4: **Association between gentamicin resistance and stress tolerance in water isolates of *Ralstonia pickettii* and *R. mannitolilytica***

Published:

Authors: Pompeyo Ferro, Ivone Vaz-Moreira and Celia M. Manaia

Year: 2019

Title: "Association between Gentamicin Resistance and Stress Tolerance in Water Isolates of *Ralstonia pickettii* and *R. mannitolilytica*"

Journal: Folia Microbiologica 64.1:63-72.

Authors contributions:

	Planning	Experimental work	Data analysis	Writting
Pompeyo Ferro	x	x	x	x
Ivone Vaz-Moreira	x		x	x
Célia M. Manaia	x		x	x

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

4.1. 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 subinhibitory 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 was observed to stimulate the biofilm formation. Subinhibitory 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.

4.2. Introduction

Members of the phylum *Proteobacteria*, mainly some families and genera of the classes *Alpha-*, *Beta-*, and *Gammaproteobacteria*, are among the most prevalent bacteria in water habitats (Vaz-Moreira *et al.,* 2014, 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, 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 has 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.

4.3.Materials and methods

4.3.1. 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 4.1). Cultures were maintained and preserved in nutritive (Luria-Bertani) broth supplemented with 15% (v/v) glycerol.

Table 4.1 . Group of isolates tested in this study

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 mM)	10^3	(Becerra-Castro *et al.,* 2015)
H2Cu5	*R. pickettii*	Hospital wastewater	Culture enrichment in modified Luria-Bertani broth with Cu^{2+} (2.5 mM)	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)

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 neighbor-joining method. The iTol software v3.2.4 (Letunic and Bork, 2016) was used to represent the isolates source in the dendrogram.

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

4.3.3. Determination of Minimum Inhibitory Concentrations (MICs).

The minimum inhibitory concentrations (MICs) were determined using the Etest or the microdilution method at 30 °C. The Etest (BioMérieux, France) or MICE (OXOID, United Kingdom) were used for the antibiotics gentamicin (CN 256–0.015 μg/mL, OXOID, MA0116F), streptomycin (SM 0.064–1024 μg/mL, BioMérieux, 526,800), ceftazidime (TZ 0.016–256 μg/mL, BioMérieux, 412293), meropenem (MEM 32–0.002 μg/mL, OXOID, MA0121F), and sulfamethoxazole (SX 0.064–1024 μg/mL, BioMérieux, 412458). The microdilution method was used for tetracycline and metals (Andrews 2001), using bacterial suspensions of absorbance 0.08–0.1 at 625 nm in Mueller-Hinton broth supplemented with 0.1–32 mg/L of tetracycline, 0.001–2 mmol/L of $NaAsO_2$, 0.01–10 mmol/L of $NiCl_2 \cdot 6H_2O$ or 1–14 mmol/L of $CuSO_4 \cdot 5H_2O$. For concentrations of $CuSO_4 \cdot 5H_2O$ above 5 mmol/L, the MICs were tested in Tris-buffered Mueller-Hinton broth. The MICs were determined as the minimum concentration that inhibited visible bacterial growth after 24 h of incubation. Based on preliminary distinctive results between the tested strains, the aminoglycoside gentamicin, and the metal arsenite were selected to assess their effects as stressors and will be from this point forward designated as stressors. Each of the five strains was assayed in stressor-free (SF) culture medium and in the presence of gentamicin or arsenite at concentrations close to the MIC value.

4.3.4. Stressors and growth kinetics.

Cultures were assayed in Mueller-Hinton broth or in this culture medium supplemented with adequate concentrations and volume of stressor solution. Therefore, strains H2Cu2, T6BT1 and L1PA1 were assayed in 125 mg/L gentamicin or 1.1 mmol/L As^{3+}; strain H2Cu5 was assayed in 6 mg/L gentamicin or 0.01mmol/L As^{3+}; and strain T6BT10 was assayed in 0.4 mg/L gentamicin or 0.01 mmol/L As^{3+}. Bacterial suspensions with an initial absorbance of 0.05 at 610 nm (A610) were incubated at 30 °C with orbital shaking (~ 70 rpm) and were monitored every hour until reached the stationary phase (~ 24 h). Growth curves and kinetic parameters (growth rate, lag phase, and yield) were determined in

triplicate in independent assays. Growth curves were represented as log values of A610 in function of time. The lag phase was the period of time necessary to start the exponential phase. The growth rate (μ) was determined based on the slope of the curve during the exponential growth phase, according to the equation Ln N_t − Ln N_0 = μ(t − t_0), where N is the number of cells at time t. The growth yield corresponded to the maximum A610 reached.

4.3.5. 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 °C) 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 *et al.,* (2007), through the calculation of a ratio A570/A620, 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 culture medium). With the procedure used, the possible contribution of the growth yield for the capacity to form biofilm was normalized by the use of the ratio A570/A620, referring to absorbance at 570 nm (measure of the biofilm formation) and absorbance at 620 nm (measure of the bacterial growth).

4.3.6. Disinfectants and inactivation

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

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

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

4.3.7. Statistical analyses

The effect of different stressors and the behavior of different strains were compared based on the parametric test one-way ANOVA or the non-parametric 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 analyses were performed with the SPSS software package, version 23.0 (IBM SPSS software, Chicago, IL).

4.4.Results

4.4.1. *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. 4.1). Although not related to the isolation origin, three phylogenetic sub-groups 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).

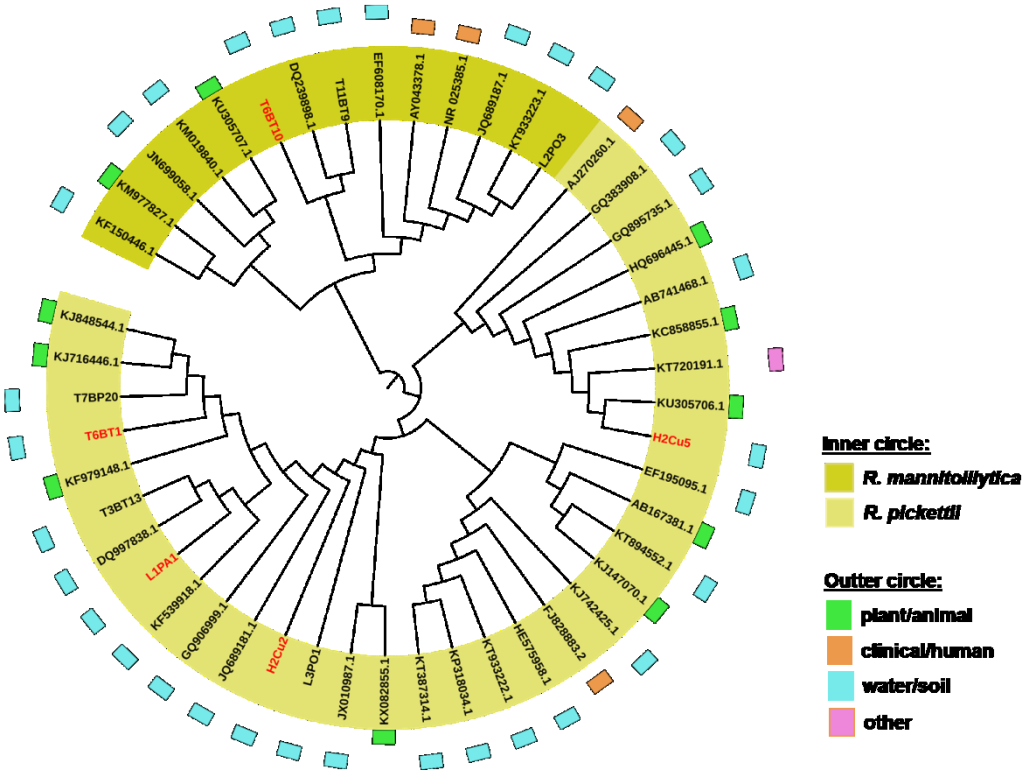


Figure 4.1 Environmental distribution of *Ralstonia pickettii* and *Ralstonia mannitolilytica*, including the strains studied (in red).

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 sub-groups, presented lower MIC values for both gentamicin and streptomycin (Table 4.2). 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.

Table 4.2 Minimum Inhibitory Concentrations (MICs) for antibiotics and metals determined 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+} (mM)**	**Ni^{2+} (mM)**	**Cu^{2+} (mM)**
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

GEN, gentamicin; STR, streptomycin ; TET, tetracycline; MER, meropenem; CEF, ceftazidime; SUL, sulfamethoxazole and metal salts of As^{3+}, Ni^{2+} and Cu^{2+}

4.4.2. Stressors and growth kinetic

Based on the hypothesis that a common mechanism of resistance could be used by these strains for gentamicin and arsenite, growth kinetic parameters were determined in the absence and in the presence of each of those stressors (Table 4.3). In the absence of any stressor, the growth rates for the five strains were similar (~ 0.4 h^{-1}) (Table 5.3). Either gentamicin or arsenite led to significant ($p < 0.05$) reductions in the growth rate, being the highest reductions observed in the presence of sub-inhibitory concentrations of gentamicin (Table 4.3). The lag phases in the absence of stressor ranged 0.7–0.9 h. In the presence of arsenite, but not in the presence of gentamicin, these values significantly ($p < 0.05$) increased (to 2.4–3.4 h) in the strains with highest MIC-As^{3+} values (Table 4.3). In absence of stressors, growth yield ranged 2.4–2.9. These values that were significantly ($p < 0.05$) reduced in the presence of arsenite for strain H2Cu2 (to 2.2) or in the presence of gentamicin for strains H2Cu2, H2Cu5, L1PA1, and T6BT1 (to 0.5–0.9). In general, the reduction of growth yield was more pronounced in the presence of gentamicin than of arsenite. These differences in the growth parameters in the presence of gentamicin or arsenite suggest that even if a common resistance mechanism is used to grow in the presence of each of those stressors, probably distinct functions are targeted in the cell by the antibiotic or the metal.

Table 4.3 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 mM As^{3+}	0.4±0.03	1;a,b	0.3±0.02	2;a	0.1±0.01	3;a	0.9±0.2	1;a	3.4±0.5	2;a	2.0±0.7	1,2;a	2.9±0.1	1;a	2.2±0.2	2;a	0.5±0.1	3;a
H2Cu5 6 mg/L GEN or 0.01 mM As^{3+}	0.3±0.01	1;a	0.3±0.04	1;a	0.1±0.01	2;b	0.7±0.4	1;a	1.0±0.3	1;b	2.2±2.5	1;a	2.5±0.3	1;a	2.0±0.3	1;a	0.9±0.2	2;a
L1PA1 125 mg/L GEN or 1.1 mM As^{3+}	0.4±0.04	1;a,b	0.3±0.04	1;a,b	0.2±0.01	2;c	0.7±0.4	1;a	3.0±0.9	2;a	0.8±0.1	1;a	2.9±0.4	1;a	2.5±0.5	1;a	0.8±0.1	2;a
T6BT1 125 mg/L GEN or 1.1 mM As^{3+}	0.4±0.02	1;a,b	0.3±0.01	2;a	0.2±0.01	3;b	0.9±0.3	1;a	2.4±0.3	2;a	1.4±0.4	1;a	2.7±0.1	1;a	2.4±0.3	1;a	0.7±0.1	2;a
T6BT10 0.4 mg/L GEN or 0.01 mM As^{3+}	0.4±0.01	1;b	0.4±0.01	1,2,b	0.4±0.01	2;d	0.8±0.1	1;a	0.8±0.1	1;b	0.7±0.1	1;a	2.4±0.5	1;a	2.1±0.4	1;a	1.9±0.6	1;b

A610, bacterial suspension absorbance at 610 nm;
Statistically significant differences between stresses 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.

4.4.3. Effect of stressors in the capacity of biofilm formation

The capacity to form biofilm may be an advantage in *Ralstonia* spp. to face adverse conditions (Adley *et al.,* 2005; Anderson *et al.,* 1990; Domenico *et al.,* 2016; Ryan *et al.,* 2011). Hence, it was hypothesized that the stressors aminoglycosides and arsenite could stimulate the capacity to produce biofilm (Fig. 4.2). The low concentrations of stressor tolerated by the aminoglycoside susceptible strains H2Cu5 and T6BT10 were not observed to induce in those strains an increased capacity to form biofilm. In contrast, the strains resistant to the aminoglycosides gentamicin and streptomycin (H2Cu2, T6BT1 and L1PA1) presented significant increases in the capacity to form biofilm, with increases of 2–4 times for the lower concentrations and 5–11 times for the highest concentrations of aminoglycosides tested, in comparison with the non-stressor assays (Fig. 4.2). In the same way, 1.1 mmol/L arsenite a significant, although lower (1.5–1.9 times), increase of biofilm formation. The capacity to form biofilm can be associated with the production of polysaccharide capsules that facilitate the adherence to surfaces and the formation of biofilms (Moxon and Kroll 1990). It was thus hypothesized that the increased capacity to form biofilm could be due to an observable overproduction of capsule polysaccharides in the presence of sub-inhibitory concentration of gentamicin. However, this hypothesis was not proved, eventually because the method used to observe capsules was not sufficiently sensitive.

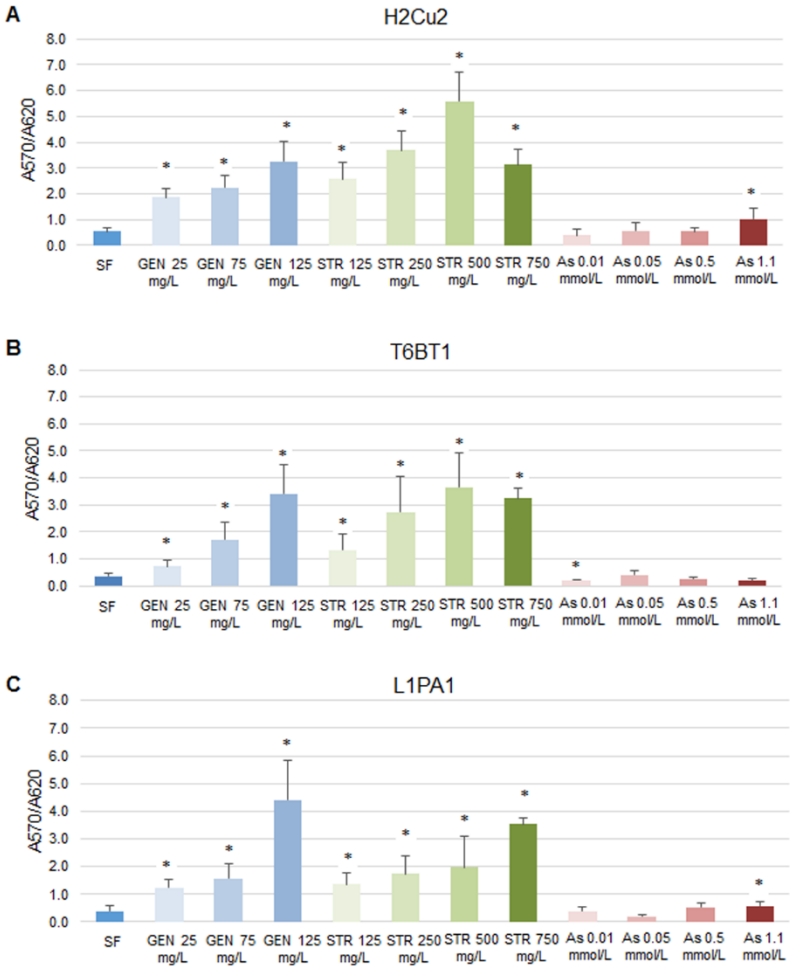


Figure 4.2 Influence of stressors on the ability of biofilm formation, for the strains H2Cu2 (A), T6BT1 (B) and L1PA1 (C).

The quantification of the biofilm formation was performed through the calculation of a ratio A_{570}/A_{620}, referring to absorvance at 570 nm (measure of the biofilm formation) and absorvance 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) 50mg/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

4.4.4. Disinfectants and inactivation

The hypothesis beyond these assays was that aminoglycoside and arsenite resistant strains would present a higher resilience against the different types of disinfectant—UV radiation, chlorine, and peroxide disinfection (Fig. 4.3). However, it was observed that only peroxide disinfection supported that hypothesis. Neither UV radiation nor chlorine were observed to produce a distinct

effect on the gentamicin resistant or susceptible strains (Fig. 4.3). The UV radiation promoted a reduction of 1–2 log at each 15 min of exposure till the maximum period tested of 45 min (Fig. 4.3a). In the presence of 5 mg/L chlorine, it was observed a sharp culture inactivation (2 min), to reach after 7 min of exposure, counts < 10 CFU/mL (Log 1) (Fig. 4.3b). 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. 4.3c).

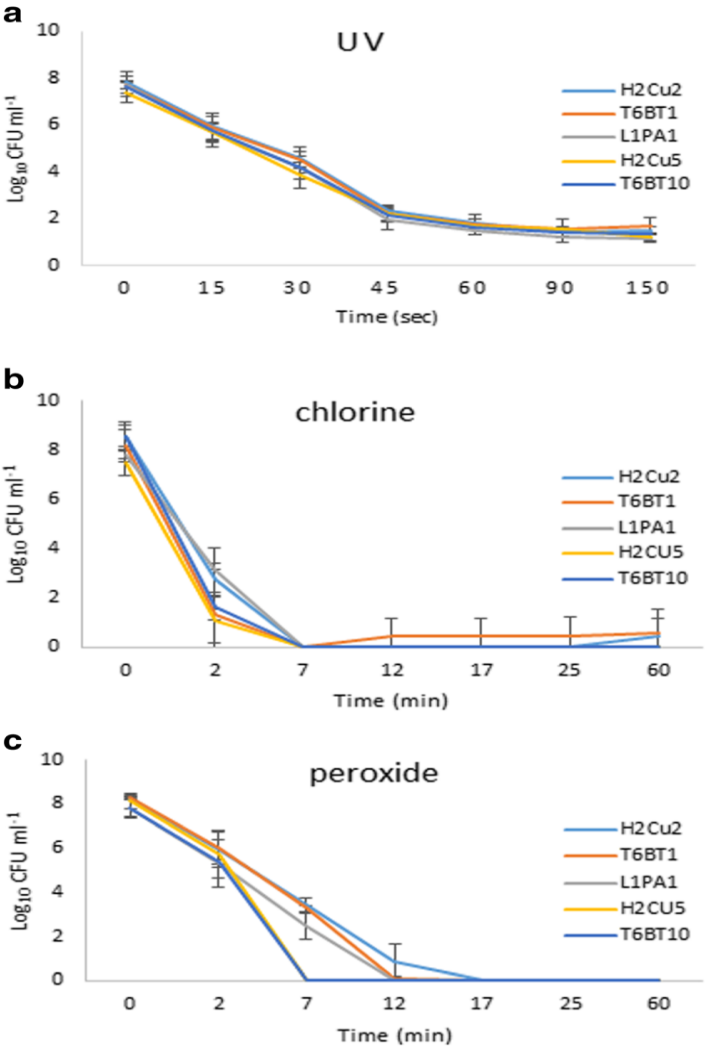


Figure 4.3 Bacterial inactivation with (a) UV radiation, (b) chlorine (5mg/L) and (c) hydrogen peroxide (0.05%).

4.5.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 (Tables 4.1 and 4.2), 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 resistance genes) or cross-resistance (same genetic determinant confers resistance to both antibiotics and metals), frequently reported for heavy metals and antibiotics (Baker-Austin *et al.,* 2006; Dib *et al.,*2008; Seiler and Berendonk, 2012; Zhou *et al.,* 2015) specially in contaminated environments (Ahemad and Malik, 2014).

Supposedly the physiological response to arsenite and aminoglycosides involves diverse mechanisms, as the response for biofilm formation and growth kinetic did not respond in the same mode for both antimicrobials (Table 4.2). However, the stress imposed by sub-inhibitory concentrations of aminoglycosides or of metals increased the capacity to form biofilms of the aminoglycoside resistant isolates (Fig. 4.2). This can be due to an unspecific stress response, not related with the resistance mechanism, in particular enhanced production of extracellular polymeric substances (EPS) which lead to cell adhesion (Baker-Austin *et al.,* 2006; Donlan and Costerton, 2002; Donlan,

2002; Lindsay and von Holy, 2006), and biofilm formation (Balaban *et al.,* 2008; Donlan and Costerton, 2002; Lindsay and von Holy, 2006). Similar results were observed for *Pseudomonas aeruginosa* and *Escherichia coli* isolates, increasing their capacity to form biofilm in the presence of aminoglycosides (Aka and Haji, 2015; Hoffman *et al.,* 2005). However, Paul *et al.,* (2014) observed a negative influence of 0.01 mmol/L arsenite on the capacity to form biofilm of *Pseudomonas* spp. or *Rhizobium* spp. strains. Some authors refer to the importance of the bacteria motility on the capacity to form biofilm (Guttenplan and Kearns, 2013; O'Toole and Kolter, 1998; Yao and Allen, 2007). Indeed, *Ralstonia* spp. are motile and this property might influence the variable capacity to form biofilm observed in the present study. However, it was not possible to assess differences in motility capabilities in the tested strains or to investigate if stressors interfere with flagella and therefore 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. 4.3). 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

4.6.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.

Chapter 5: **Hints for acquired gentamicin and arsenite resistance in *Ralstonia pickettii* water isolates**

In preparation for submission:

Authors: Pompeyo Ferro, Ivone Vaz-Moreira and Celia M. Manaia

Title: "Hints for acquired gentamicin and arsenite resistance in *Ralstonia pickettii* water isolates"

Authors contributions:

	Planning	Experimental work	Data analysis	Writting	Bioinformatics analysis
Pompeyo Ferro	x	x	x	x	
Ivone Vaz-Moreira	x	x	x	x	x
Célia M. Manaia	x		x	x	

5. Hints for acquired gentamicin and arsenite resistance in *Ralstonia pickettii* water isolates.

5.1. Abstract

Ralstonia pickettii are ubiquitous in water environments, from mineral water to wastewater. Although observed in most *R. pickettii* isolates, suggesting its intrinsic character, genatmicin resistance, presumably associated with increased arsenite tolerance, is not common to all species members. The current work aimed to investigate the possible association between gentamicine and arsenite tolerance and unveil possible molecular mechanisms. The study included 37 *R. pickettii* isolates from drinking mineral (n=14) and tap water (n=17), and hospital wastewater (n=6), identified based on 16S rRNA gene sequence analysis. The strains were characterized for gentamicin and arsenite minimum inhibitory concentration (MIC), the number and size of plasmids and presence of genetic elements associated with arsenite tolerance, Integrative and Conjugative Elements (ICEs), among others.

The gentamicin resistant (GR, n=33) and susceptible (GS, n=4, all from hospital effluent) strains clustered apart based on the 16S rRNA gene sequence analysis, a division that, except for one strain, coincided with arsenite MIC values of 1.4 mM (n=32) and 0.05 mM (n=5), respectively. In all GR isolates with increased arsenite tolerance (n=32), but not in the others, were observed ICE- and *ars* operon-related genes. Moreover, the *cmeA* gene related with an RND efflux pump, detected in all strains, was conserved in each subset of GR and GS isolates. These findings suggested the evolutionary divergence of both GR and GS lineages, which might explain the distinct phenotypes. The comparative analyses of the whole genome sequences of one GR and one GS isolate, suggested that some genes only detected in the GR isolate as well as some mutations observed between both are good candidates of the divergent evolution of both phenotypes. Although ICEs and *ars* operon presence suggested that GR and arsenite tolerance was once acquired, other genome features favor a divergent evolutionary path for both lineages.

5.2. Introduction

Members of the genus *Ralstonia*, within the class *Betaproteobacteria*, in particular the species *Ralstonia pickettii* are ubiquitous in aquatic habitats, specifically 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 believed to be 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 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 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, for example: to 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 and 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.,* 2015), the current knowledge about the nature of antibiotic resistance, including if it is acquired or intrinsic is scarce (Ryan and 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. Given the fact that aminoglycosides and arsenite had distinct effects on growth kinetics, cross-resistance, meaning a common resistance mechanism, was discarded by Ferro *et al.,* (2018). However, co-resistance due to genetic linkage might explain the co-occurrence of both phenotypes. This hypothesis drove this study, which aim was to investigate the phenotypic response to aminoglycoside and arsenite in a larger number of wastewater and drinking mineral and tap water *R. pickettii* isolates, and explore putatively associated genetic elements.

5.3. Materials and methods

5.3.1. 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 5.1) (Becerra-Castro *et al.,* 2015; Falcone-Dias *et al.,* 2012; Vaz-Moreira *et al.,* 2013). Cultures were cultivated on Plate Count Agar (PCA) and preserved in nutritive (Luria-Bertani) broth supplemented with 15% (v/v) glycerol. The strains identification was made based on the 16S rRNA gene sequence analysis using the primers 27F and 1492R (Lane, 1991) and species identification by comparison with the public database EzBioCloud (Yoon *et al.,* 2017). The 16S rRNA gene based phylogenetic comparison included all isolates and five other *R. pickettii* strains whose gene sequence was available in the GenBank, using the Mega7 software (Kumar *et al.,* 2016).

Table 5.1 *Ralstonia pickettii* isolates tested in this study.

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)

5.3.2. 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 ug; nalidixic acid NA, 30 ug), 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 ≤ 12, S ≥ 15; AK30: R ≤ 14, S ≥ 17; K30: R ≤ 13, S ≥ 18; N10: R ≤ 12, S ≥ 15; NET30: R ≤ 12, S ≥ 15; TOB10: R ≤ 12, S ≥ 15; STR10: R ≤ 11, S ≥ 15; MER10: R ≤ 15, S ≥ 19; CP30: R ≤ 14, S ≥ 18; CEF30: R ≤ 14, S ≥ 18; TIC75: R ≤ 15, S ≥ 24; CT50: R ≤ 10, S ≥ 11; CIP5: R ≤ 15, S ≥ 21; NA30: R ≤ 13, S ≥ 19; SUL25: R ≤ 12, S ≥ 17; SXT25: R ≤ 10, S ≥ 16; TET30: R ≤ 11, S ≥ 15. In each assay, the reference strain *Pseudomonas aeruginosa* DSM 1117 was used as quality control.

The minimum inhibitory concentrations (MICs), defined as the minimum concentration that inhibited visible bacterial growth after 24 h of incubation at 30 °C, 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. For the microdilution assays were used

bacterial suspensions with absorbance at 610 nm of 0.08–0.1 in Mueller-Hinton broth supplemented with 0.001–2 mM of $NaAsO_2$.

5.3.3. Screening of selected genetic determinants

Based on the comparative analysis of the GR and GS genomes and on the literature, were screened genetic elements related with: 1) antibiotic resistance (beta-lactamase genes bla_{OXA-22}, and bla_{OXA-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).

The beta-lactamase genes bla_{OXA-22} and bla_{OXA-60}, frequently described for *Ralstonia* spp. (Girlich *et al.,* 2006) and detected in the H2Cu2 and H2Cu5 genomes (Vaz-Moreira *et al.,* 2016), were screened for all the strains and the nucleotide sequence determined for representative GR and GS strains using the primers and annealing temperatures listed in Table 2. Two of the genes identified in the *ars* operon, the genes *arsH* and *acr3*, associated to arsenite resistance by detoxification (Yang and Rosen, 2016), were screened using the primers and annealing temperatures listed in Table 5.2.

Table 5.2 List of primers used in this study.

Genes	Fragment size (bp)	Primers	Annealing temperature (°C)	Reference
16S	1465	27F: 5'-GAG TTT GAT CCT GGC TCA G-3'. 1492R: 5'-TAC CTT GTT ACG ACT T-3'	55	(Lane, 1991)
int	1035	intFor TTTCATTTCACCATGACTCCAG. intRev GAGAGCAGTCGATAGGCTTCC	61	(Ryan *et al.,* 2009)
traG	1483	traGF GTTCGAGTGGTGGTTCTTCTTC. traGR GAAATTGCTGTCCGCGTAGTAG	61	
repA	911	repA_Ral_fw GCGACTACCAGCGCCTCAAG. repA_Ral_rev GTGTGGTCATGGAGACTTCTCC	52	This study
trbI	1505	trbI_Ral_fw ATTACTCCAAGCTGCCGAAG. trbI_Ral_rev CCGCTGTCAAGTCCTTCATT	55	This study
cirIm	~220	RE1 GCATGGAAGACTTGACAG. LE1 GAGCTTGAGTTTTGCCACG	52	(Ryan *et al.,* 2009)
***bla*$_{OXA-22}$**	206	OXA22_fw TTTCCTCAAAGACGAGCACA. OXA22_rev CTACGGCAACATGGATGTGA	53	This study
***bla*$_{OXA-60}$**	324	OXA60_fw AGTCTGATCGCCTTCGACAC. OXA60_rev CTTCACCGGCAACTGCTT	60	This study
cmeA	1195	*cme*A_F TTGTTCCGTCGTTACCGTTC. cmeA_R GGGCGGTCGATAAAGAAGC.	54	This study
acr3	997	ACR3_fw ATCACGCTTGGTGCTCGT. ACR3_rev GTATGGGTGCTGTTGTGCAT	52	This study
arsH	965	arsH_fw: GTGGATTGCTTCCTCTCCAG. arsH_rev:_AGCATCCCATCCTCATCAAC	50	This study

The *cmeA* gene belonging to an RND efflux system was screened for all the strains and nucleotide sequenced determined in representative GR and GS strains, using the primers and annealing temperatures listed in Table 5.2. The deduced amino acid sequences of the corresponding membrane fusion protein were compared in GR and GS strains, looking for possible mutations. The prediction of a possible effect of the aminoacids changes in the protein structure was tested using the PredictProtein 2013 website (https://www.predictprotein.org/). Sequences alignment and analysis was done with Mega7 software (Kumar *et al.,* 2016).

The number and size of plasmids was determined by Pulsed-Field Gel Electrophoresis (PFGE), as described before (Ferreira *et al.,* 2019). Briefly, DNA plugs were digested with S1 nuclease for 30 min at 37 ºC and the number and size of plasmids determined by PFGE using a 1% SeaKem Gold agarose gel run in CHEF III DR System (Bio-Rad, Laboratories, Hercules, CA, United States) with

0.5x TBE for 18 h at 14 ºC, with an initial switch time of 6.8 s and final switch time of 35.4 s, 6 V, with a 120º angle. For size determination was used the *Salmonella enterica* serovar Braenderup H9812 (ATCC) DNA digested with XbaI as a reference size standard (Magalhães *et al.,* 2015).

For ICEs detection and characterization, a group of five genes (*int*, *traG*, *repA*, *trbI* and cirlm), were screened for all strains using conventional PCR with specific primers and conditions (Table 5.2) recommended by Ryan *et al.,* (2009) or optimized for this study.

5.3.4. Comparative genome analysis

The genomes of two hospital wastewater isolates, one GR (strain H2Cu2) and one GS (strain H2Cu5), were compared aiming the identification of genetic determinants possibly related with distinct gentamicin and arsenite resistance phenotypes. The Ion Torrent PGM genome sequences are deposited in DDBJ/ENA/GenBank under the accession numbers MCGA00000000 and MCGB00000000, respectively (Vaz-Moreira *et al.,* 2016). A function-based comparison was made using the gene prediction and annotation obtained from RAST server (http://rast.nmpdr.org/rast.cgi). The analysis targeted the categories "membrane transport", "stress response", "virulence, disease and defense", and "phages, prophages, transposable elements, and plasmids", assuming that are those possibly related with resistance phenotypes. The comparison relied on the search of genetic determinants present only in the GR strain or on the detection of point mutations that distinguished both genomes. The latter compared a set of genes (n=77) which annotation included the keywords: "RND", "efflux", "secretion", "metal", "antibiotic", and "multidrug". Once established the list of genes of interest, those genes were compared for both strains based on the nucleotide sequences, using the blastall tool from SAMtools (Li *et al.,* 2009), and a functional comparison was also done recurring to the blastp tool (https://blast.ncbi.nlm.nih.gov) and the percentage of amino acids similarity calculated, as well as the number of possible non-conservative mutations. The search for acquired antimicrobial resistance genes was done using the ResFinder 3.1 search tool (Zankari *et al.,* 2012) with a threshold

value of 90%, and plasmids were searched using the PlasmidFinder 2.0 tool (Carattoli *et al.,* 2014). Considering the observed co-occurrence of gentamicin and arsenite tolerance, it was done a search for arsenite resistance related genes in both genomes annotation and then was done the prediction of the *ars* operon in the GR strain using the Operon mapper tool (http://biocomputo.ibt.unam.mx/operon_mapper/) (Taboada *et al.,* 2018). In addition, the ICEfinder tool available in the ICEberg 2.0 database webpage (M. Liu *et al.,* 2019) was used to search for ICEs and IMEs (Integrative and mobilizable elements) in the two genomes. The presence of insertion sequences (IS) was tested using the ISfinder (https://isfinder.biotoul.fr/).

5.4. Results and discussion

5.4.1. Phylogenetic and phenotypic characterization of the *Ralstonia pickettii* strains

The 37 *R. pickettii* strains isolated from wastewater, tap water, and mineral water were resistant to the antibiotics streptomycin and colistin sulphate and susceptible to tetracycline, sulfamethoxazole, sulfamethoxazole/trimethoprim, ciprofloxacin and cephalothin. In contrast to what was observed for the aminoglycoside streptomycin, gentamicin resistance was not common to all isolates. Most of the strains (33 out of the 37), isolated from wastewater, tap water and mineral water were resistant to gentamicin (GR) (Table 5.3), in contrast with four wastewater isolates that were susceptible to gentamicin (GS). With the exception of one tap water isolate, T9CP10, all the GR strains also presented an increased tolerance to arsenite, with MIC values for gentamicin of >256 mg/L and of 1.4 mM for arsenite. Strain T9CP10 had the same MIC for gentamicin (>256 mg/L) and a low MIC value for arsenite (0.05 mM). GS strains presented MIC values of 4-8 mg/L for gentamicin and of 0.05 mM for arsenite..

The comparative 16S rRNA gene sequence analysis showed that the 42 sequences had sequence identity values ranging from 97.1% to 100%. This analysis evidenced different clusters, one including all GS strains (100% sequence identity), another all GR strains (100% sequence identity), except T9CP10 (99.8%

sequence identity with GS and 99.9% with GR) and two unclustered database strains (98.5-98.6% sequence identity with GS and 98.3-98.5% with GR) (Figure 5.1). This analysis suggested that GR and increased arsenite tolerance might be due to intrinsic resistance mechanisms associated with the strain phylogeny.

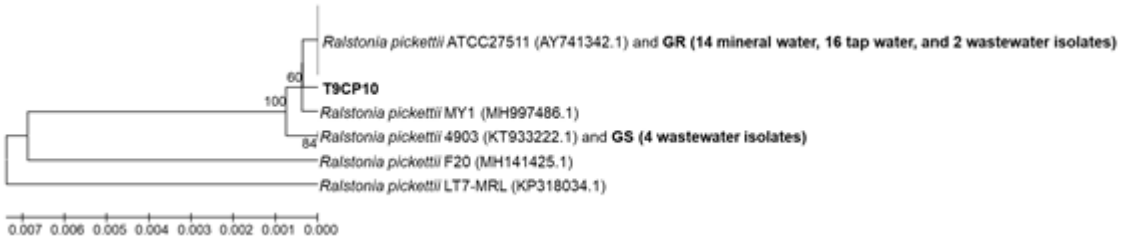


Figure 5.1 Phylogenetic relationship among the *Ralstonia pickettii* strains used in this study and five *Ralstonia pickettii* reference strains available in public databases, based on the analysis of 1380 bp of the 16S rRNA gene.

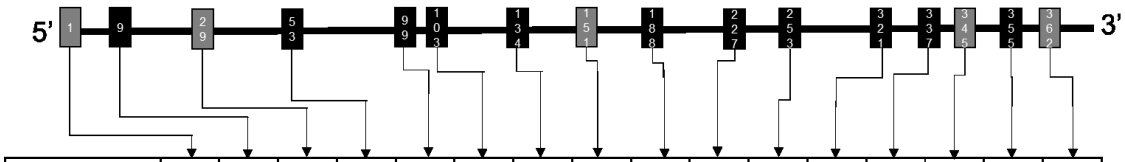
The dendrogram was inferred using the UPGMA method in MEGA7 (Kumar *et al.,* 2016). GR, gentamicin and arsenite resistant strains; GS, gentamicin and arsenite susceptible strains.

5.4.2. Analyses of genetic determinants

As expected according to literature (Girlich *et al.,* 2004; Girlich *et al.,* 2006; Nordmann *et al.,* 2000), all the *R. pickettii* strains presented the genes $bla_{\text{OXA-22}}$ and $bla_{\text{OXA-60}}$, conferring resistance to beta-lactam antibiotics. A comparison of the genes nucleotide sequences for some GR and GS isolates showed the existence of two types of sequence, in agreement with what was observed for the 16S rRNA gene (data not shown).

The genes *arsH* and *acr3,* components of the *ars* operon, were detected only in the strains with increased tolerance to arsenite - all the GR strains with exception of T9CP10 (Table 5.3). The presence of efflux pumps, such as the RND (Resistance-Nodulation-Division) efflux pumps, a family of antibiotics and chemotherapeutic agents transporters widespread among Gram-negative bacteria (Nikaido and Takatsuka, 2009) was also explored through the amplification of the membrane fusion protein encoding gene *cmeA*. As expected, due to the widespread presence of this type of efflux pumps, the gene was detected in all the *R. pickettii* strains, although with distinct deduced amino acid sequences. Our

hypothesis was that a possible mutation in this protein could be associated with the GR, for that reason were analysed the sequences of a group of GR and GS strains. In total were observed 16 aminoacid mutations (Figure 5.2), five were conservative mutations that might not have a relevant effect in the protein structure. The other 11 mutations were non-conservative and observed for all the GR strains, in strain T9CP10, and could probably be associated with a gentamicin resistance. In GR strains, the presence of glycine (position 9), alanine (positions 134, 253 and 355), proline (position 188), lysine (position 227), arginine (position 321), and serine (position 337), contrasted with the observation of alanine, serine or threonine, threonine, glutamine, glutamine, and glycine in the same positions in GS strains (Figure 5.2). The tap water GR strain T9CP10 differed from all the others, GR and GS strains, in the position 99, with a threonine instead of an alanine. The presence of a such a number of amino acid mutations in a fragment of 362 amino acid residues may have an important effect in the protein structure, mainly in the region of positions 188 and 337 where five non-conservative mutations were observed. According to the PredictProtein website that allows the prediction of protein function and structure based in the amino acid sequence, the mutations observed in the GR strain promoted the alteration of some of the predicted binding sites, mainly in that region (positions 188-337) where a higher number of non-conservative mutations was observed (data not shown). A change in the protein structure may modify the type of molecules able to be transported by the channel. These findinds would need to be validated.



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

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

Figure 5.2 Schematic representation of the aminoacid sequence of the RND efflux pump related gene *cmeA*, with indication of the positions of the possible mutations.
In grey are indicated the conservative mutations and in black the non-conservative mutations.

Based on the PFGE analyses, a single plasmid was detected in each GR strain. Specifically, mineral water isolates had plasmids with 244 kbp or 180 kbp, with the plasmid size difference conciding with the mineral water batch. Most of the tap water isolates also had a plasmid with 180 kbp, with exception of the strain T9CP10, with a plasmid with 260 kbp, and the strain T6BT1 in which was not possible to detect any plasmid. In wastewater isolates was observed the presence of a single plasmid in each of the two GR strains, and in the GS strains were detected two plasmids, one with 75 kbp and another with 244 kbp or 260 kbp (Table 5.3). The number and size of plasmids seemed to be more related with the phylogeny and environment of origin than to the observed resistance phenotype. Nevertheless, this does not exclude the possibility that these plasmids harbor some genetic elements related with the tolerance to gentamicin or arsenite.

Genetic determinants associated with the mobile genetic elements ICEs were screened based on PCR detection, being observed an association between the presence of the target genes and an increased tolerance to arsenite. The genes related with ICEs were detected in all the strains with an arsenite MIC of 1.4 mM (Table 5.3).

Table 5.3 Phenotypic and genotypic characterisation of *the Ralstonia pickettii* strains concerning their tolerance to antibiotics and arsenite, and the presence of the genetic elements ICEs, plasmids and the arsenite resistance related genes *acr3* and *arsH*.

Source	Strains	Antibiotic resistance phenotypes						MICs		ICEs	Plasmid size (kpb)	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	-	-

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

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

5.4.3. Genetic comparison among an gentamicin resistant and susceptible strain

The analyses of genetic determinants evidenced the formation of two major groups, one comprised by GR strains harbouring one plasmid and genes related with ICEs and arsenite resistance, and another of GS strains harboring two plasmids and missing the genetic determinants related with ICEs and arsenite resistance (Table 5.3) and both groups showing distinct nucleotide sequences in specific genes. Based on the conserved pattern of each group, was selected one strain of each to proceed with whole genome sequence analysis. The comparison of the two genomes, one belonging to a GR strain and the other to a GS strain, was a major approach used for screening genetic elements putatively associated with the distinct phenotypes of gentamicin and arsenite resistance. This analysis was focused on membrane transport, stress response, virulence, disease and defense, and phages, prophages, transposable elements, and plasmids, since these were the functional categories that might include determinants associated with both resistance phenotypes. Two criteria of screening were used, the search for genes present only in the GR strain and the search for point mutations in genes present in both GR and GS strains. Genes putatively encoding functions that were only detected in the GR strain (Table 5.4), include a set of proteins related with type IV, VI and VII secretion systems. Type IV secretion systems (T4SS) form a membrane-spanning secretion channel and often an extracellular component such as a pilus, that will allow the transport of DNA among cells (M. Liu *et al.,* 2019; Wozniak and Waldor, 2010). Indeed, T4SS have been associated with antibiotic resistance acquisition in *Betaproteobacteria*, which may happen by mediation of DNA transfer or through DNA-uptake and -release systems (Cascales and Christie, 2013). In addition, T4SS are frequently responsible for the ICEs DNA transfer in Gram-negative bacteria. The type VI secretion systems (T6SS), important for pathogenesis and bacterial competition, are broadly distributed in *Proteobacteria* with roles associated with translocation of toxic effector proteins into prokaryotic cells (Costa *et al.,* 2015). Zhang *et al.,* (2011) demonstrated the association of T6SS with an increased resistance of *Pseudomonas aeruginosa* biofilms to some antibiotics. However, the major function described for T6SS 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) . Curioulsy, this secretion systems are not described in *Proteobacteria*. However, in the GR strain H2Cu2 were annotated two genes as part of a T7SS related with the formation of a pili and chaperone protein. Therefore, the presence of those two genes does not indicate that the bacteria have a T7SS, because many other genes are lacking. Another important feature only detected in the GR strain was the *ars* operon, not detected in the GS strain H2Cu5. In addition, the GR strain harbours some genes related with TRAP, Ton and Tol transporters, the uptake of selenate and selenite, mechanisms of regulation of the osmotic stress, lysozyme inhibitors, and some phage capsid and tail proteins that are not present in the GS strain (Table 5.4).

For the category of phages, prophages, transposable elements, and plasmids were not detected proteins common to both strains, H2Cu2 and H2Cu5. Within the other three functional classes analyzed, it was observed that 65 out of 77 proteins common to GR and GS strains had point mutations. These could be good candidates to explain phenotypic differences, in contrast with 12 proteins that had 100% amino acid sequence identity. The proteins presenting a higher percentage of dissimilarity, mainly considering non-conservative mutations, were those with a higher probability of suffer a change in the protein structure. Defining a cut-off of 4% occurrence of non-conservative mutations, 10 proteins were in this situation, most of them related with metals tolerance (copper, zinc, chromium and mercury) and secretion systems (Table 5.5). The *cmeA* gene related with the RND efflux pump had a percentage of dissimilarity of 2.9, below the 4% threshold defined. 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 deserve further investigation to try to understand the

possible effect of the observed mutations in the strain's tolerance to metals or antimicrobials.

Table 5.4 Genes only present in the genome of the gentamicin resistant strain H2Cu2.

Category	**Subcategory**	**Subsystem (role or proteins)**
Membrane Transport	Protein and nucleoprotein secretion system, Type IV	Conjugative transfer (TrbC, TrbL)
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)
Membrane Transport	Protein secretion system, Type VII (Chaperone/Usher pathway, CU)	Type 1 pili (mannose-sensitive fimbriae, gamma-fimbriae FimA)
Membrane Transport	Protein secretion system, Type VII (Chaperone/Usher pathway, CU)	Sigma-Fimbriae chaperone, tip adhesin and usher protein
Membrane Transport	TRAP transporters	TRAP Transporter collection (TRAP-type C4-dicarboxylate transport system, large permease and periplasmic component)
Membrane Transport	no subcategory	Ton and Tol transport systems (TPR domain protein, putative component of TonB system and TonB-dependent receptor)
Stress Response	Detoxification	Uptake of selenate and selenite (various polyols ABC transporter: ATP-binding component; periplasmic substrate-binding protein; permease component 1 and 2)
Stress Response	Osmotic stress	Choline and Betaine Uptake and Betaine Biosynthesis (Choline-sulfatase)
Virulence, Disease and Defense	Resistance to antibiotics and toxic compounds	Arsenic resistance (ArsH, ArsA, ArsD, Acr3)
Virulence, Disease and Defense	Resistance to antibiotics and toxic compounds	Lysozyme inhibitors (Inhibitor of vertebrate lysozyme precursor)
Phages, Prophages, Transposable elements, Plasmids	Phages, Prophages	Phage capsid proteins (scaffolding protein, phage head completion-stabilization protein, major capsid protein)
Phages, Prophages, Transposable elements, Plasmids	Phages, Prophages	Phage tail fiber proteins
Phages, Prophages, Transposable elements, Plasmids	Phages, Prophages	Phage tail proteins (major tube protein, completion protein, length tape-measure protein)

Table 5.5 Comparison of functional categories for GR and GS strains H2Cu2 and H2Cu5.

Category	Subcategory	Subsystem/Role	Nucleotides similarity (%)	Aminoacid similarity (%)	Matches/total aminoacids	non-conservative /total mutations	non-conservative mutations/total aminoacids (%)
Virulence, Disease and Defense	Bacteriocins, ribosomally synthesized antibacterial peptides	Tolerance to colicin E2 (CreA)	92.1	95.6	152/159	1/7	1/159 (0.6)
	Resistance to antibiotics and toxic compounds	Beta-lactamase class D (OXA-60)	90.0	94.0	234/249	8/15	8/249 (3.2)
		Beta-lactamase class C	92.4	96.7	407/421	7/14	7/421 (1.7)
		Beta-lactamase class D (OXA-22)	94.5	95.6	262/274	6/12	6/274 (2.2)
		Metal-dependent beta-lactamase	90.7	94.6	247/261	9/14	9/261 (3.4)
		Cobalt-zinc-cadmium resistance (Cation efflux system protein CusA)	91.6	97.1	1001/1031	15/30	15/1031 (1.5)
		Cobalt-zinc-cadmium resistance	87.4	96.1	367/382	3/15	3/382 (0.8)
		Cobalt-zinc-cadmium resistance (membrane fusion protein, CzcB family)	**100.0**	**100.0**	520/520	n.a.	(0)
		Cobalt-zinc-cadmium resistance (Copper sensory histidine kinase CusS)	**100.0**	**100.0**	465/465	n.a.	(0)
		Cobalt-zinc-cadmium resistance (regulator CusR)	**100.0**	**100.0**	228/228	n.a.	(0)
		Cobalt-zinc-cadmium resistance (RND efflux outer membrane protein, CzcC family)	96.4	98.2	435/443	4/8	4/443 (0.9)
		Copper homeostasis (CopG protein)	83.9	86.2	94/109	7/15	7/109 **(6.4)**
		Copper homeostasis	90.1	94.2	65/69	2/4	2/69 (2.9)
		Copper homeostasis (CopC)	80.8	81.3	104/128	15/24	15/128 **(11.7)**
		Copper homeostasis (histidine kinase CusS)	**100.0**	**100.0**	465/465	n.a.	(0)
		Copper homeostasis (TolC family protein)	92.1	93.6	440/470	17/30	17/470 (3.6)
		Copper homeostasis (response regulator CusR)	**100.0**	**100.0**	228/228	n.a.	(0)
		Copper homeostasis (FUSC family protein)	93.3	97.4	719/738	8/19	8/738 (1.1)

		Copper homeostasis (Cu(I)-responsive transcriptional regulator)	93.3	95.4	145/152	4/7	4/152 (2.6)
		Copper homeostasis (copper oxidase)	96.2	98.6	426/432	3/6	3/432 (0.7)
		Copper homeostasis (Multidrug resistance transporter, Bcr/CflA family)	87.5	89.9	214/238	16/24	16/238 **(6.7)**
		Copper homeostasis: copper tolerance (protein CutE)	92.8	97.0	488/503	7/15	7/503 (1.4)
		Copper homeostasis: copper tolerance (protein CorC)	92.5	97.7	514/526	4/12	4/526 (0.8)
		Mercuric reductase (MerA)	99.9	99.8	560/561	1/1	1/561 (0.2)
		Mercury resistance operon (co-regulator MerD)	**100.0**	**100.0**	121/121	n.a.	(0)
		Mercury resistance operon (MerR family transcriptional regulator)	92.3	93.2	124/133	6/9	6/133 **(4.5)**
		Mercury resistance operon (MerE)	**100.0**	**100.0**	78/78	n.a.	(0)
		Mercury resistance operon (MerT)	92.0	**100.0**	116/116	n.a.	(0)
		Mercury resistance operon (MerP)	**100.0**	**100.0**	91/91	n.a.	(0)
		Multidrug Resistance, Tripartite Systems Found in Gram Negative Bacteria (DHA2 family efflux MFS transporter permease subunit)	88.5	94.8	491/518	10/27	10/518 (1.9)
		Multidrug Resistance, Tripartite Systems Found in Gram Negative Bacteria (HlyD family efflux transporter periplasmic adaptor subunit)	92.2	97.9	420/429	5/9	5/429 (1.2)
		Multidrug Resistance, Tripartite Systems Found in Gram Negative Bacteria	92.9	96.1	490/510	14/20	14/510 (2.7)
		Multidrug Resistance Efflux Pumps (Acriflavin resistance protein)	95.8	99.3	1032/1039	4/7	4/1039 (0.4)
		Multidrug Resistance Efflux Pumps (MATE family efflux transporter)	93.5	96.7	433/448	8/15	8/448 (1.8)
		Multidrug Resistance Efflux Pumps (LysR family transcriptional regulator)	93.7	99.0	311/314	3/3	3/314 (1.0)
		Multidrug Resistance Efflux Pumps (RND efflux system, inner membrane transporter CmeB)	96.2	98.0	977/997	11/20	11/997 (1.1)
		Multidrug Resistance Efflux Pumps (RND efflux system, membrane fusion protein CmeA)	91.3	95.0	357/374	11/16	11/374 (2.9)
		Multidrug Resistance Efflux Pumps (RND efflux system, outer membrane lipoprotein CmeC)	88.7	93.6	486/519	*	*

		Multidrug Resistance Efflux Pumps (RND efflux system, outer membrane lipoprotein, NodT family)	91.8	97.4	488/501	6/13	6/501 (1.2)
		Multidrug Resistance Efflux Pumps (acrAB operon, TetR (AcrR) family)	92.4	95.5	213/223	6/10	6/223 (2.7)
		Multidrug Resistance Efflux Pumps (Type I secretion outer membrane protein, TolC precursor)	92.5	97.2	452/465	9/13	9/465 (1.9)
		Resistance to chromium compounds (ChrB)	91.4	94.5	310/328	9/18	9/328 (2.7)
		Resistance to chromium compounds (ChrA)	86.6	90.9	359/395	19/36	19/395 **(4.8)**
		Resistance to fluoroquinolones (DNA gyrase subunit A)	92.2	98.3	874/889	9/15	9/889 (1.0)
		Resistance to fluoroquinolones (DNA gyrase subunit B)	96.2	96.9	816/842	15/26	15/842 (1.8)
		Resistance to fluoroquinolones (Topoisomerase IV subunit A)	95.6	99.2	774/780	1/6	1/780 (0.1)
		Resistance to fluoroquinolones (Topoisomerase IV subunit B)	95.3	99.1	655/661	2/6	2/661 (0.3)
Stress Response	Heat shock	Heat shock dnaK gene cluster extended (Probable Fe(2+)-trafficking protein YggX)	96.4	**100.0**	91/91	n.a.	(0)
	Oxidative stress	Oxidative stress (Ferric uptake regulation protein FUR)	92.0	90.7	137/151	8/14	8/151 **(5.3)**
		Oxidative stress (Ferroxidase)	96.0	98.8	164/166	0/2	(0)
		Oxidative stress (Hydrogen peroxide-inducible genes activator)	91.3	98.3	289/294	1/5	1/294 (0.3)
		Oxidative stress (Iron-binding ferritin-like antioxidant protein)	96.0	98.8	164/166	0/2	(0)
		Oxidative stress (Zinc uptake regulation protein ZUR)	92.4	92.0	161/175	10/14	10/175 **(5.7)**
		Protection from Reactive Oxygen Species (Superoxide dismutase [Cu-Zn] precursor)	94.6	96.7	177/183	2/6	2/183 (1.1)
		Protection from Reactive Oxygen Species (Superoxide dismutase [Fe])	98.6	**100.0**	192/192	n.a.	(0)
Membrane Transport	Cation transporters	Copper Transport System (Copper-translocating P-type ATPase)	93.3	97.4	719/738	8/19	8/738 (1.1)
		Magnesium and cobalt efflux protein (CorC)	92.5	97.7	514/526	4/12	4/526 (0.8)
		Magnesium and cobalt transport protein (CorA)	96.1	99.7	320/321	1/1	1/321 (0.3)
		Magnesium transport (ATPase protein C)	**100.0**	**100.0**	98/98	n.a.	(0)
		Transport of Nickel and Cobalt (HupE-UreJ family metal transporter)	92.7	94.6	194/205	6/11	6/205 (2.9)

	Protein secretion system, Type II	DNA gyrase inhibitor YacG	94.1	97.0	65/67	1/2	1/67 (1.5)
		General Secretion Pathway (protein C)	89.6	91.6	208/227	14/19	14/227 **(6.2)**
		General Secretion Pathway (protein GspE)	95.5	99.0	513/518	3/5	3/518 (0.6)
		General Secretion Pathway (protein GspG)	95.0	97.5	155/159	3/4	3/159 (1.9)
		General Secretion Pathway (protein GspH)	92.5	93.9	154/164	4/10	4/164 (2.4)
		General Secretion Pathway (protein GspI)	92.3	97.6	121/124	9/13	9/124 **(7.3)**
		General Secretion Pathway (protein GspJ)	94.5	96.0	237/247	9/10	9/247 (3.6)
		General Secretion Pathway (protein GspK)	92.8	91.7	332/362	14/30	14/30 (3.9)
		General Secretion Pathway (protein GspL)	89.5	94.1	444/472	19/28	19/472 **(4.0)**
		General Secretion Pathway (protein GspM)	93.9	96.6	168/174	4/6	4/174 (2.3)
		General Secretion Pathway (protein N)	92.1	92.9	260/280	11/20	11/280 (3.9)
		Widespread colonization island (CpaF family protein)	92.3	98.9	446/451	1/5	1/451 (0.2)
		Widespread colonization island (pilus assembly protein)	89.1	96.4	431/447	10/16	10/447 (2.2)
		Widespread colonization island (Type II/IV secretion system protein TadC, associated with Flp pilus assembly)	91.4	96.7	318/329	5/11	5/318 (1.5)
		Widespread colonization island (Type II/IV secretion system secretin RcpA/CpaC, associated with Flp pilus assembly)	86.5	91.7	407/444	19/37	19/444 **(4.3)**
	no subcategory	Ton and Tol transport systems (TonB)	96.7	94.5	206/218	5/12	5/218 (2.3)
		Ton and Tol transport systems (TolC precursor)	92.5	97.2	452/465	9/13	9/465 (1.9)

*low nucleotides similarity avoiding a good comparison between aminoacid sequences

The search in the genomes for antimicrobial resistance genes using Resfinder revealed only the presence of two beta-lactamases, $bla_{\text{OXA-22}}$ and $bla_{\text{OXA-60}}$, the same that were already screened for all the isolates. Using this database, no genes related with gentamicin or aminoglycosides resistance were identified in the genomes.

As stated above a group of genes related with arsenic resistance were detected only in the GR strain genome (Table 5.4). This group of genes, and some others, form a functional *ars* operon, with approximately 7 kbp, with the structure described in Figure 5.3. The *ars* operon includes seven identified coding genes: *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) (Fekih *et al.,* 2018). Although arsenite MIC values are not available in the literature for *Ralstonia picketttii*, for Acr3 transporter positive *Pseudomonas* spp. those values were of 1.75-7 mM (Achour *et al*., 2007; Cai *et al*., 2009), higher than those determined for the arsenite resistant *R. pickettii* (1.4 mM, Table 5.3). However, most of those *Pseudomonas* spp. strains harbour a second transporter (ArsB) together with the Acr3, which can contribute to raise the MIC value. The GS strain H2Cu5 harboured only two genes that could be related with arsenite resistance, the arsenate reductase (*arsC*) and the arsenical pump membrane protein (*arsB*). The ArsB pump could confer some resistance to arsenite. However, ArsB pump needs to be energized by ATP hydrolysis (catalysed by *arsA*) or by motive force in arsRBC operons (Fekih *et al.,* 2018), and none of those genes (*arsA* or *arsR*+*arsC*) was detected in the GS strain genome.

Figure 5.3 *Ralstonia picketti* H2Cu2 *ars* operon prediction.

The genetic environment of *ars* operon in the GR strain genome suggested the vicinity of mobile genetic elements. Motivated by the observed association

between arsenite resistance and the presence of the ICEs genetic determinants (Table 5.3), the tool ICEfinder was used to screen ICEs related genes in the contig where *ars* operon was detected. This search revealed the presence of a putative ICE close to the *ars* operon, about 12 kbp upstream, identified by the ICEfinder as similar to an ICE detected in the *Achromobacter xylosoxidans* A8 (score 41). Although it is not possible to prove the inclusion of the *ars* operon in the identified ICE structure, the neighborhood of both suggest that genetic linkage. A second putative ICE was identified by the ICEfinder in the GR strain genome. This ICE was similar to that reported in the *R. pickettii* 12J (score of 39) by Ryan *et al*. (2009), used in this study to design the ICE screening PCR primers (Table 5.2). By the analysis of the GR strain genome was not possible to identify the presence of cargo genes in the structure of the ICE related with the resistance to aminoglycosides. 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 he GS strain H2Cu5were detected in the genome some elements that typically form an ICE, such as the relaxase, and some T4SS elements, some others were missing.

The search for plasmids in the GR and GS genomes using the Plasmidfinder tool did not allow the detection of any plasmid. However, Plasmidfinder database only includes plasmids from *Enterobacteriaceae* and Gram-positive organisms, which may justify this result.

5.5. Conclusions

- Besides to aminoglycosides, resistance to beta-lactams and colistin was frequent in *R. pickettii*, isolated from aquatic environments.
- Tolerance to gentamicin and arsenite are related with the phylogeny of the strains.
- The presence of the *ars* operon is associated with the increased tolerance to arsenite and to the presence of mobilizable ICEs, but no association was found with gentamicin resistance.
- Elements such as T6SS, may be associated with the acquisition of gentamicin and arsenite resistance, being observed only in the GR strain.

- Both GR and GS *R. pickettii* strains harbors plasmids, although no relationship with gentamicin and arsenite resistance was foreseen.

The analysis of the phylogeny, phenotypes and genotypes of a set of *R. pickettii* from different aquatic environments allowed us to conclude 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.

6. General Discussion

Within the urban water cycle, drinking water is that which quality may have the highest direct impacts on human health and wellbeing. In contrast, wastewater may represent the major source of bacteria with harmful characteristics, such as virulence or antibiotic resistance that may be spread in the environment. Therefore, the study of ubiquitous bacteria capable of thriving in the distinct compartments of the urban water cycle may shed some light on the ecology and adaptation potential in according to specific genetic traits. Being *Betaproteobacteria* among the most abundant and common components of the drinking water microbiome, in both treated water and in natural mineral/spring water, also ubiquitous in other habitats (Figure 3.1), we were interested in exploring their diversity, and virulence and resistance genes. The diversity survey revealed that *Betaproteobacteria* genera include bacteria that can be also found in the human microbiome. Whereas such association may be innocuous to humans, in some cases of debilitated hosts, human health risks can be anticipated, mainly if it is considered the common resistance to disinfection, the presence of virulence factors and occurrence of intrinsic or acquired antibiotic resistance. All these are factors that may increase the probability of causing opportunistic infections. Some of the *Betaproteobacteria* features that explain the good fitness of these bacteria in water habitats, are those that may facilitate the colonization and invasion of an opportunistic infection.

Betaproteobacteria genera commonly found in drinking water include *Achromobacter, Burkholderia*, and *Ralstonia*. *Ralstonia* spp. have been recently often reported as a nosocomial infectious organism, being the low frequency of infection episodes reported in the past attributed to misidentifications of these bacteria (Coman *et al.,* 2017; Daxboeck *et al.,* 2005; Ryan and Adley, 2014; Ryan *et al.,* 2006). *Ralstonia* spp. was selected as a drinking water *Betaproteobacteria* group that deserves further investigation and which investigation was furthered in this study. The association between gentamicin resistance and arsenite or stress tolerance in water isolates of *Ralstonia pickettii* and *R. mannitolilytica* (Chapter 4) was an interesting hint to proceed with the investigation in this bacterial genus. It was important to explore the association between gentamicin resistance and arsenite tolerance, which could be an indication of co-resistance (genetic linkage between two or more

resistance genes) or cross-resistance (same genetic determinant confering resistance to both antibiotics and metals), frequently reported for heavy metals and antibiotics (Baker-Austin *et al.,* 2006; Dib *et al.,* 2008; Seiler and Berendonk, 2012; Zhou *et al.,* 2015). Probably, different genetic mechanisms are involved in both types of tolerance, as was suggested by distinct kinetic growth responses. The hypothesis of genetic linkage could not be proved or refuted. However, the investigation made with a larger number of isolates, would confirm that only gentamicin resistant and arsenite tolerant strains yielded integrative and conjugative elements (ICEs) and the *arsH* and *acr3* genes, part of an *ars* operon, conferring resistance to arsenite (Chapter 5). These results favor both the genetic linkage hypothesis and the acquisition of these traits through ICEs. Thus, we confirmed the presence of ICEs in gentamicin resistamt *Ralstonia pickettii,* coinciding with what was previously reported (Ryan *et al.,* 2009), and it was possible detect the *ars* operon downstream an integrase-regulator gene and about 12 kbp upstream the predicted ICE. Although plasmids were detected in most of the *R. pickettii* strains, both gentamicin resistant and susceptible, it was not possible to establish an association between the presence of these mobile genetic elements and the resistance to gentamicin. Even though, the observed association between resistance to gentamicin and increased tolerance to arsenite, seems to be a consequence of the *R. pickettii* phylogeny, indicating a probable co-evolution of the two mechanisms of resistance. Interestingly, one of the gentamicin resistant *R. pickettii* strains analysed (T9CP10) seems to be a transitory state in the evolution of these lineages. This strain has a resistance phenotype to gentamicin but not an increased tolerance to arsenite, and phylogenetically is also distinguishable from the other gentamicin resistant strains. Further studies of the genome of this strain can bring important clues to continue to understanding the association between gentamicin and arsenite resistance observed for *R. pickettii*. In addition, the study of the genetic composition of the plasmids detected in most of the *R. pickettii* strains should also give important information regarding the resistance phenotypes observed. Being *R. pickettii* an important colonizer of "clean environments", the understanding of these resistance mechanisms, as well as others that confer them the ability to survive to disinfection processes, may be of high relevance for the public health.

7. Main Conclusions

Some *Betaprotebacteria* are ubiquitous in treated water and in natural mineral water/spring water both as planktonic or biofilm bacteria. The bacterial genera: *Achromobacter, Burkholderia* and *Ralstonia*, previously reported as causing outbreaks of infection are among the *Betaproteobacteria* most commonly found in drinking water and are worthy of further ecology and genetics driven research.

Ralstonia pickettii and *Ralstonia mannitolilytica* displayed resistance to antibiotics belonging to different classes (aminoglycosides, beta-lactams, carbapenems and colistin), being gentamicin resistance observed in most but not in all isolates.

Biofilm formation was stimulated in the presence of sub-inhibitory concentrations of aminoglycosides or arsenite, in the gentamicin-resistant strains but not in the susceptible strains of *Ralstonia* spp.

Hydrogen peroxide inactivation was slower in the gentamicin-resistant strains but not in the susceptible strains of *Ralstonia* spp., while no differences were observed for hypochlorite or ultraviolet radiation.

Gentamicin resistance was associated with increased tolerance to arsenite in *Ralstonia* spp.

Gentamicin and arsenite-resistant *Ralstonia pickettii* strains, in contrasts with the gentamicin and arsenite-susceptible strains, have ICEs related genes and genes of the arsenic operon (*arsH* and *acr3*), hinting possible gene acquisition events in the gentamicin resistant lineage. These findings were supported by the fact that 77 genes encoding proteins associated with the functional categories "membrane transport", "stress response" and "virulence, disease and defense", were detected in

the genome sequence of gentamicin and arsenite-resistant *R. pickettii* strain H2Cu2 but not in the gentamicin and arsenite-susceptible *R. pickettii* strain H2Cu5. The opposite, meaning genes that were detected in strain H2Cu2 but not in H2Cu5, corresponded to 30 genes in the same three categories.

Gentamicin and arsenite-resistant and gentamicin and arsenite-susceptible *R. pickettii* strains differed in their 16S rRNA, $bla_{\text{OXA-22}}$, $bla_{\text{OXA-60}}$ and *cmeA* genes sequences, suggesting that they represent lineages with distinct evolutionary paths., as could be confirmed based on the amino acid sequences associated with the functional categories.

8. Suggestions of Future Work

Conduct studies of horizontal gene transfer in other bacteria that inhabit aquatic environments of clinical interest, such as the genera: *Burkholderia*, *Curvibacter* and *Cupriavidus*, since by sharing the same aquatic habitats and the human microbiome, the possibility that they are capable of genetic exchange cannot be ruled out.

Extend the whole genome analyses to more isolates, in particular strain T9CP10, which displays gentamicin but not arsenite resistance, and that it was suggested to belong to a lineage distinct from the two major groups studied.

Explore the ICEs, as well as plasmids, structure and activity in the isolates analysed in this study and in isolates of distinct origins and taxonomic groups.

9. References

Abbott, I. J., & Peleg, A. Y. (2015). *Stenotrophomonas, Achromobacter,* and *Nonmelioid Burkholderia* Species: Antimicrobial Resistance and Therapeutic Strategies. *Semin Respir Crit Care Med*, *36*, 99–110.

Abdallah, M. A., Gey, N., DiGiuseppe, P., Cox, J., Luirink, J., Appelmelk, B., & Bitter, W. (2007). The ESX-5 Secretion system of *Mycobacteria marinum* Modulates the Macrophage Response. *The Journal of Immunology*, *5*, 883–891. https://doi.org/10.4049/jimmunol.181.10.7166

Abhishek, K. S., Kombade, S. P., & Nag, V. L. (2018). Rare Cases of *Ralstonia pickettii* Associated with Tonsillitis and Wound Infection. *Journal of Medical Bacteriology*, *7*(3, 4), 1–4.

Abreu, A. G., & Barbosa, A. S. (2017). How *Escherichia coli* Circumvent Complement-Mediated Killing. *Frontiers in Inmunology*, *8*(April), 1–6. https://doi.org/10.3389/fimmu.2017.00452

Achour, A. R., Bauda, P., & Billard, P. (2007). Diversity of arsenite transporter genes from arsenic-resistant soil bacteria. *Research in Microbiology*, *158*(2), 128–137. https://doi.org/10.1016/j.resmic.2006.11.006

Adley, C. C., Ryan, M. P., Pembroke, J. T., & Saieb, F. M. (2005). *Ralstonia picketii* : biofilm formation in high purity water. *Biochemistry*, 151–162.

Ahemad, M., & Malik, A. (2014). Prevalence of heavy metal and antibiotic resistance in bacterial isolates from metal polluted soils. *Microbiol. J., 4*(1), 12–21.

Aka, S. T., & Haji, S. H. (2015). Sub-MIC of antibiotics induced biofilm formation of *Pseudomonas aeruginosa* in the presence of chlorhexidine. *Brazilian Journal of Microbiology*, *46*(1), 149–154. https://doi.org/10.1590/S1517-838246120140218

Alanis, A. J. (2005). Resistance to antibiotics: Are we in the post-antibiotic era? *Archives of Medical Research*, *36*, 697–705. https://doi.org/10.1016/j.arcmed.2005.06.009

Alasehir, E. A., Ipek, B. O., Thomas, D. T., Sitar, M. E., & Ercan, T. E. (2018). *Ralstonia insidiosa* Neonatal Sepsis : A Case Report and Review of the Literature.

Journal of Pediatric Infectious Diseases.

Allen, H. K., Donato, J., Wang, H. H., Cloud-Hansen, K. a, Davies, J., & Handelsman, J. (2010). Call of the wild: antibiotic resistance genes in natural environments. *Nature Reviews. Microbiology*, *8*(4), 251–259. https://doi.org/10.1038/nrmicro2312

Almuzara, M., Limansky, A., Ballerini, V., Galanternik, L., Famiglietti, A., & Vay, C. (2010). In vitro susceptibility of *Achromobacter* spp. isolates: comparison of disk diffusion, Etest and agar dilution methods. *International Journal of Antimicrobial Agents*, *35*(1), 68–71. https://doi.org/10.1016/j.ijantimicag.2009.08.015

Alonso, A., Sánchez, P., & Martínez, J. L. (2001). Environmental selection of antibiotic resistance genes. *Environmental Microbiology*, *3*, 1–9. https://doi.org/10.1046/j.1462-2920.2001.00161.x

Alves De Brito, C. F., Carvalho, C. M. B., Santos, F. R., Gazzinelli, R. T., Oliveira, S. C., Azevedo, V., & Teixeira, S. M. R. (2004). *Chromobacterium violaceum* genome: Molecular mechanisms associated with pathogenicity. *Genetics and Molecular Research*, *3*(1), 148–161.

Amadou, C., Mangenot, S., Glew, M., Bontemps, C., Capela, D., Dossat, C., … Masson-boivin, C. (2008). Genome sequence of the B-rhizobium *Cupriavidus taiwanensis* and comparative genomics of rhizobia. *Genome Research*, *18*, 1472–1483. https://doi.org/10.1101/gr.076448.108.7

Amoureux, L., Bador, J., Siebor, E., Taillefumier, N., Fanton, A., & Neuwirth, C. (2013). Epidemiology and resistance of *Achromobacter xylosoxidans* from cystic fibrosis patients in Dijon , Burgundy : First French data. *Journal of Cystic Fibrosis*, *12*(2), 170–176. https://doi.org/10.1016/j.jcf.2012.08.005

Anderson, R. L., Holland, B. W., Carr, J. K., Bond, W. W., & Favero, M. S. (1990). Effect of disinfectants on pseudomonads colonized on the interior surface of the PVC pipes. *American Journal of Public Health*, *80*(1), 17–21. https://doi.org/10.2105/AJPH.80.1.17

Andrews, J. M. (2001). Determination of minimum inhibitory concentrations. *The Journal of Antimicrobial Chemotherapy*, *48 Suppl 1*, 5–16. Retrieved from

http://www.ncbi.nlm.nih.gov/pubmed/11420333

Araya, R., Tani, K., Takagi, T., Yamaguchi, N., & Nasu, M. (2003). Bacterial activity and community composition in stream water and biofilm from an urban river determined by fluorescent in situ hybridization and DGGE analysis Ruben. *FEMS Microbiology Ecology*, *43*, 111–119.

Armstrong, J. L., Calomiris, J. O. N. J., & Seidler, R. J. (1982). Selection of Antibiotic-Resistant Standard Plate Count Bacteria During Water Treatmentt. *Applied and Environmental Microbiology*, *44*(2), 308–316.

Aydin, S., Ince, B., & Ince, O. (2015). Development of antibiotic resistance genes in microbial communities during long-term operation of anaerobic reactors in the treatment of pharmaceutical wastewater. *Water Research*, *83*, 337–344. https://doi.org/10.1016/j.watres.2015.07.007

Bador, J., Amoureux, L., Blanc, E., & Neuwirth, C. (2013). Innate Aminoglycoside Resistance of *Achromobacter xylosoxidans* Is Due to AxyXY-OprZ, an RND-Type Multidrug Efflux Pump. *Antimicrobial Agents and Chemotherapy*, *57*(1), 603–605. https://doi.org/10.1128/AAC.01243-12

Bahar, O., Goffer, T., & Burdman, S. (2009). Type IV Pili are required for virulence, twitching motility, and biofilm formation of *acidovorax avenae* subsp. *Citrulli. Molecular Plant-Microbe Interactions : MPMI*, *22*(8), 909–920. https://doi.org/10.1094/MPMI-22-8-0909

Baker-Austin, C., Wright, M. S., Stepanauskas, R., & McArthur, J. V. (2006). Co-selection of antibiotic and metal resistance. *Trends in Microbiology*, *14*(4), 176–182. https://doi.org/10.1016/j.tim.2006.02.006

Balaban, N., Ren, D., Givskov, M., & Bovbjerg, R. (2008). Introduction. In J. W. Costerton (Ed.), In: Springer series on biofilms. Control of Biofilm Infection by Signal Manipulation (Volume 2, pp. 1–11). Los Angeles, CA USA. https://doi.org/10.1007/978-3-540-73853-4

Baldani, J. I., Baldani, V. L. D., & Dobereiner, J. (2005). Genus III. *Herbaspirillum* Baldani, Baldani, Seldin and Dobereiner 1986a, 90VP emend. Baldani, Pot, Kirchhof, Falsen, baldani, Olivares, Hoste, Kersters, Hartmann, Gillis and

Dobereiner 1996, 808. In G. M. Garrity, D. J. Brenner, N. R. Krieg, & J. T. Staley (Eds.), Bergey's Manual of Systematic Bacteriology (vol 2, 2ed, pp. 629–636). New York: Springer.

Balder, R., Lipski, S., Lazarus, J. J., Grose, W., Wooten, R. M., Hogan, R. J., … Lafontaine, E. R. (2010). Identification of *Burkholderia mallei* and *Burkholderia pseudomallei* adhesins for human respiratory epithelial cells. *BMC Microbiology*, *10*(250), 1–20.

Baldermann, C., Lupas, A., Lubienecki, J., & Engelhardt, H. (1998). The regulated outer membrane protein Omp21 from *Comamonas acidovorans* is identified as a member of a new Family of eight-stranded B-Sheet Proteins by its sequence and properties. *Journal of Bacteriology*, *180*(15), 3741–3749. http://ukpmc.ac.uk/abstract/MED/9683466

Baldwin, A., Sokol, P. A., Parkhill, J., & Mahenthiralingam, E. (2004). The *Burkholderia cepacia* Epidemic Strain Marker Is Part of A Novel Genomic Island Encoding Both Virulence and Metabolism-Associated Genes in *Burkholderia cenocepacia*. *Infection and Immunity*, *72*(3), 1537–1547. https://doi.org/10.1128/IAI.72.3.1537-1547.2004

Baquero, F., Martínez, J. L., & Cantón, R. (2008). Antibiotics and antibiotic resistance in water environments. *Current Opinion in Biotechnology*, *19*, 260–265. https://doi.org/10.1016/j.copbio.2008.05.006

Barrell, R. A. E., Hunter, P. R., Nichols, G., Barrell, R. A. E., & Hunter, P. R. (2000). Microbiological standards for water and their relationship to health risk. *Communicable Disease and Public Health*, *3*(1), 8–13.

Bartram, J., Cortruvo, J., Exner, M., Fricker, C., & Glasmacher, A. (2003). Heterotrophic Plate Counts and Drinking-water Safety. Cornwall UK: IWA Publishing.

Batte, M., Appenzeller, B., Fass, S., Gauthier, V., Jorand, F., Mathieu, L., … J-c, B. (2003). Biofilms in drinking water distribution systems. Summe School BIO-IMEBII, Galway, Irland.

Baxter, I. A., Lambert, P. A., & Simpson, I. N. (1997). Isolation from clinical sources of

Burkholderia cepacia possessing characteristics of *Burkholderia gladioli*. *Journal of Antimicrobial Chemotherapy*, *39*, 169–175.

Becerra-Castro, C., Macedo, G., Silva, A. M. T., Manaia, C. M., & Nunes, O. C. (2016). Science of the Total Environment *Proteobacteria* become predominant during regrowth after water disinfection. *Science of the Total Environment*, *573*, 313–323. https://doi.org/10.1016/j.scitotenv.2016.08.054

Becerra-Castro, C., Machado, R. A., Vaz-Moreira, I., & Manaia, C. M. (2015). Assessment of copper and zinc salts as selectors of antibiotic resistance in Gram-negative bacteria. *Science of the Total Environment*, *530–531*. https://doi.org/10.1016/j.scitotenv.2015.05.102

Bernier, S. P., Nguyen, D. T., Sokol, P. A., & Tn, A. (2008). A LysR-Type Transcriptional Regulator in *Burkholderia cenocepacia* Influences Colony Morphology and Virulence. *Infection and Immunity*, *76*(1), 38–47. https://doi.org/10.1128/IAI.00874-07

Berry, D., Xi, C., & Raskin, L. (2006). Microbial ecology of drinking water distribution systems. *Current Opinion in Biotechnology*, *17*, 297–302. https://doi.org/10.1016/j.copbio.2006.05.007

Bhatt, G., & Denny, T. P. (2004). *Ralstonia solanacearum* iron scavenging by the siderophore staphyloferrin B is controlled by PhcA, the global virulence regulator. *Journal of Bacteriology*, *186*(23), 7896–7904. https://doi.org/10.1128/JB.186.23.7896-7904.2004

Bi, D., Xu, Z., Harrison, E. M., Tai, C., Wei, Y., He, X., … Ou, H. Y. (2011). ICEberg: A web-based resource for integrative and conjugative elements found in Bacteria. *Nucleic Acids Research*, 1–6. https://doi.org/10.1093/nar/gkr846

Biggins, J. B., Kang, H., Ternei, M. A., Deshazer, D., & Brady, S. F. (2014). The Chemical Arsenal of *Burkholderia pseudomallei* Is Essential for Pathogenicity. *Journal of the American Chemical Society*, *136*, 9484–9490. https://doi.org/10.1021/ja504617n

Birlutiu, R. M., Roman, M. D., Cismasiu, R. S., Fleaca, S. R., & Popa, C. M. (2017). Sonication contribution to identifying prosthetic joint infection with *Ralstonia*

pickettii : a case report and review of the literature. *BMC Musculosketal Disorders*, (2), 1–6. https://doi.org/10.1186/s12891-017-1678-y

Blair, J. M. A., Webber, M. A., Baylay, A. J., Ogbolu, D. O., & Piddock, L. J. V. (2014). Molecular mechanisms of antibiotic resistance. *Nature Review Microbiology,* 46*bl*, 1–10. https://doi.org/10.1038/nrmicro3380

Boattini, M., Bianco, G., Biancone, L., Cavallo, R., Costa, C., & Boattini, M. (2018). *Ralstonia mannitolilytica* bacteraemia : a case report and literature review. *Le Infezioni in Medicina*, *4*, 374–378.

Boon, C., Deng, Y., Wang, L. H., He, Y., Xu, J. L., Fan, Y., … Zhang, L. H. (2008). A novel DSF-like signal from *Burkholderia cenocepacia* interferes with Candida albicans morphological transition. *ISME Journal*, *2*(1), 27–36. https://doi.org/10.1038/ismej.2007.76

Borgeaud, S., Metzger, L. C., Scrignari, T., & Blokesch, M. (2015). The type VI secretion system of *Vibrio cholerae* fosters horizontal gene transfer. *Scientific Reports*, *347*.

Boutros, N., Gonullu, N., Casetta, A., Guibert, M., Ingrand, D., & Lebrun, L. (2002). *Ralstonia pickettii* traced in blood culture bottles. *Journal of Clinical Microbiology*, *40*(7), 2666–2667. https://doi.org/10.1128/JCM.40.7.2666-2667.2002

Brown, S. P., Cornforth, D. M., & Mideo, N. (2012). Evolution of virulence in opportunistic pathogens: generalism, plasticity, and control. *Trends in Microbiology*, *20*(7), 336–342. https://doi.org/10.1016/j.tim.2012.04.005

Brumbley, S. M., Carney, B. F., & Denny, T. P. (1993). Phenotype Conversion in *Pseudomonas solanacearum* Due to Spontaneous Inactivation of PhcA, a Putative LysR Transcriptional Regulator. *Journal of Bacteriology*, *175*(17), 5477–5487.

Burdman, S., & Walcott, R. (2012). *Acidovorax citrulli*: Generating basic and applied knowledge to tackle a global threat to the cucurbit industry. *Molecular Plant Pathology*, *13*(8), 805–815. https://doi.org/10.1111/j.1364-3703.2012.00810.x

Buroni, S., Pasca, M. R., Flannagan, R. S., Bazzini, S., Milano, A., Bertani, I., … Riccardi, G. (2009). Assessment of three Resistance-Nodulation-Cell Division

drug efflux transporters of *Burkholderia cenocepacia* in intrinsic antibiotic resistance. *BMC Microbiology*, *11*, 1–11. https://doi.org/10.1186/1471-2180-9-200

Burrus, V., & Waldor, M. K. (2004). Shaping bacterial genomes with integrative and conjugative elements. *Research in Microbiology*, *155*(5), 376–386. https://doi.org/10.1016/j.resmic.2004.01.012

Burtnick, M. N., Brett, P. J., & Deshazer, D. (2014). Proteomic Analysis of the *Burkholderia pseudomallei* Type II Secretome Reveals Hydrolytic Enzymes , Novel Proteins , and the Deubiquitinase TssM. *Infection and Immunity*, *82*(8), 3214–3226. https://doi.org/10.1128/IAI.01739-14

Butt, A., Higman, V. A., Williams, C., Crump, M. P., Hemsley, C. M., Harmer, N., & Titball, R. W. (2014). The HicA toxin from *Burkholderia pseudomallei* has a role in persister cell formation. *Biochemistry*, *344*(19464), 333–344. https://doi.org/10.1042/BJ20140073

Cai, L., Liu, G., Rensing, C., & Wang, G. (2009). Genes involved in arsenic transformation and resistance associated with different levels of arsenic-contaminated soils. *BMC Microbiology*, *9*, 4. https://doi.org/10.1186/1471-2180-9-4

Calero-Caceres, W., Javier, M., Martín-díaz, J., & Muniesa, M. (2017). The occurrence of antibiotic resistance genes in a Mediterranean river and their persistence in the riverbed sediment *. *Environmental Pollution*, *223*, 384–394. https://doi.org/10.1016/j.envpol.2017.01.035

Camargo De Souza, D., Palmeiro, J. K., Maestri, A. C., Cogo, L. L., Rauen, C. H., Graaf, M. E., … Nogueira, S. (2018). Case Report *Ralstonia mannitolilytica* bacteremia in a neonatal intensive care unit. *Revista Da Sociedade Brasileira de Medicina Tropical*, *51*(5), 709–711. https://doi.org/10.1590/0037-8682-0118-2018

Campos, C. G., Borst, L., & Cotter, P. A. (2013). Characterization of BcaA, a Putative Classical Autotransporter Protein in *Burkholderia pseudomallei. Infection and Immunity*, *81*(4), 1121–1128. https://doi.org/10.1128/iai.01453-12

Cantón, R., & Ruiz-Garbajosa, P. (2011). Co-resistance: An opportunity for the bacteria and resistance genes. *Current Opinion in Pharmacology*, *11*, 477–485.

https://doi.org/10.1016/j.coph.2011.07.007

Carattoli, A., Zankari, E., Garciá-Fernández, A., Larsen, M. V., Lund, O., Villa, L., … Hasman, H. (2014). In Silico detection and typing of plasmids using plasmidfinder and plasmid multilocus sequence typing. *Antimicrobial Agents and Chemotherapy*, *58*(7), 3895–3903. https://doi.org/10.1128/AAC.02412-14

Cascales, E., & Christie, P. J. (2013). The versatile bacterial type IV secretion systems. *Nature Reviews. Microbiology*, *1*(2), 1–28. https://doi.org/10.1038/nrmicro753.THE

CDC. (1998). Nosocomial *Ralstonia pickettii* Colonization Associated With Intrinsically Contaminated Saline Solution — Los Angeles , California , 1998 Corneal Decompensation After Intraocular Ophthalmic Surgery — Miss. *Centers for Disease Control and Prevention, Atlanta USA*, 4–7.

Chan, Y. Y., & Chua, K. L. (2005). The *Burkholderia pseudomallei* BpeAB-OprB efflux pump: Expression and impact on quorum sensing and virulence. *Journal of Bacteriology*, *187*(14), 4707–4719. https://doi.org/10.1128/JB.187.14.4707-4719.2005

Chao, Y., Yang, Y., Ju, F., Zhang, X., Wu, W., & Zhang, T. (2013). Metagenomic analysis reveals significant changes of microbial compositions and protective functions during drinking water treatment. *Scientific Reports*, *3*, 1–9. https://doi.org/10.1038/srep03550

Chapman, J. S. (2003). Disinfectant resistance mechanisms, cross-resistance, and co-resistance. *International Biodeterioration and Biodegradation*, *51*(4), 271–276. https://doi.org/10.1016/S0964-8305(03)00044-1

Chen, Y., Huang, W., Chen, C., Sun, S., & Kuo, F. (2017). An Outbreak of *Ralstonia pickettii* Bloodstream Infection Associated with an Intrinsically Contaminated Normal Saline Solution. *Infection Control & Hospital Epidemiology*, *38*(4). https://doi.org/10.1017/ice.2016.327

Chua, K. L., Chan, Y. Y., & Gan, Y. H. (2003). Flagella Are Virulence Determinants of *Burkholderia pseudomallei*. *Infection and Immunity*, *71*(4), 1622–1629. https://doi.org/10.1128/IAI.71.4.1622

CLSI. (2015). *M100-S25* Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fifth Informational Supplement.

Coenye, T., Vandamme, P., & Lipuma, J. J. (2002). Infection by *Ralstonia* species in cystic fibrosis patients: Identification of *R. pickettii* and *R. mannitolilytica* by polymerase chain reaction. *Emerging Infectious Diseases*, *8*(7), 692–696. https://doi.org/10.3201/eid0807.010472

Colburn-Clifford, J., & Allen, C. (2010). A cbb(3)-type cytochrome C oxidase contributes to *Ralstonia solanacearum* R3bv2 growth in microaerobic environments and to bacterial wilt disease development in tomato. *Molecular Plant-Microbe Interactions : MPMI*, *23*(8), 1042–1052. https://doi.org/10.1094/MPMI-23-8-1042

Coman, I., Bilodeau, L., Lavoie, A., Carricart, M., Tremblay, F., Zlosnik, J. E., & Berthiaume, Y. (2017). *Ralstonia mannitolilytica* in cystic fibrosis: A new predictor of worse outcomes. *Respiratory Medicine Case Reports*, *20*, 2016–2018. https://doi.org/10.1016/j.rmcr.2016.11.014

Cornelis, G. R. (2006). The type III secretion injectisome. *Nature Reviews. Microbiology*, *4*(December 2006). https://doi.org/10.1038/nrmicro1526

Costa, T. R. D., Felisberto-rodrigues, C., Meir, A., Prevost, M. S., Redzej, A., Trokter, M., & Waksman, G. (2015). Secretion systems in Gram-negative bacteria: structural and mechanistic insights. *Nature Reviews. Microbiology*, *13*(6), 343–359. https://doi.org/10.1038/nrmicro3456

Council directive 98/83/EC. (1998). COUNCIL DIRECTIVE 98/83/EC of the quality of water intended for human consumption as amended by regulation 1882/2003/EC. (Vol. 31).

Cox, G., & Wright, G. D. (2013). Intrinsic antibiotic resistance: Mechanisms, origins, challenges and solutions. *International Journal of Medical Microbiology*, 6. https://doi.org/10.1016/j.ijmm.2013.02.009

Cruz-Migoni, A., Hautbergue, G. M., Artymiuk, P. J., Baker, P. J., Bokori-Brown, M., Chang, C. Te, … Rice, D. W. (2011). A *Burkholderia pseudomallei* toxin inhibits helicase activity of translation factor eIF4A. *Science, American Association for the*

Advancenment of Sicience, *334*(6057), 821–824. https://doi.org/10.1126/science.1211915

Cuccui, J., Milne, T. S., Harmer, N., George, A. J., Harding, S. V., Dean, R. E., … Prior, J. L. (2012). Characterization of the *Burkholderia pseudomallei* K96243 Capsular Polysaccharide I Coding Region. *Infection and Immunity*, *80*(3), 1209–1221. https://doi.org/10.1128/iai.05805-11

Cullinane, M., Gong, L., Li, X., Adler, N., Tra, T., Prescott, M., … Prescott, M. (2008). Stimulation of autophagy suppresses the intracellular survival of *Burkholderia pseudomallei* in mammalian cell lines. *Autophagy*, *4*(6), 744–753. https://doi.org/10.4161/auto.6246

Davies, J., & Davies, D. (2010). Origins and Evolution of Antibiotic Resistance. *Microbiol. Mol. Biol. Rev.*, *74*(3), 417–433. https://doi.org/10.1128/mmbr.00016-10

Daxboeck, F., Stadler, M., Assadian, O., Marko, E., Hirschl, A. M., & Koller, W. (2005). Characterization of clinically isolated *Ralstonia mannitolilytica* strains using random amplification of polymorphic DNA (RAPD) typing and antimicrobial sensitivity, and comparison of the classification efficacy of phenotypic and genotypic assays. *Journal of Medical Microbiology*, *54*, 55–61. https://doi.org/10.1099/jmm.0.45656-0

De Baere, T., Steyaert, S., Wauters, G., De Vos, P., Goris, J., Coenye, T., … Vaneechoutte, M. (2001). Classification of *Ralstonia pickettii* biovar 3/' thomasii' strains (pickett 1994) and of new isolates related to nosocomial recurrent meningitis as *Ralstonia mannitolytica* sp. nov. *International Journal of Systematic and Evolutionary Microbiology*, *51*(2), 547–558. https://doi.org/10.1099/00207713-51-2-547

Delafont, V., Bouchon, D., Hechard, Y., & Moulin, L. (2016). Environmental factors shaping cultured free-living amoebae and their associated bacterial community within drinking water network. *Water Research*, (May). https://doi.org/10.1016/j.watres.2016.05.044

Delavat, F., Miyazaki, R., Carraro, N., Pradervand, N., & Meer, J. R. Van Der. (2017). The hidden life of integrative and conjugative. *FEMS Microbiology Reviews*, *41*,

512–537. https://doi.org/10.1093/femsre/fux008

DeShazer, D., Brett, P. J., & Woods, D. E. (1998). The type II O-antigenic polysaccharide moiety of *Burkholderia pseudomallei* lipopolysaccharide is required for serum resistance and virulence. *Molecular Microbiology*, *30*(5), 1081–1100. https://doi.org/10.1046/j.1365-2958.1998.01139.x

Dias, M. F., Reis, M. P., Acurcio, L. B., Carmo, A. O., Diamantino, C. F., Motta, A. M., … Nascimento, A. M. A. (2018). Changes in mouse gut bacterial community in response to different types of drinking water. *Water Research*, (2018). https://doi.org/10.1016/j.watres.2017.12.052

Dib, J., Motok, J., Zenoff, V. F., Ordoñez, O., & Farías, M. E. (2008). Occurrence of resistance to antibiotics, UV-B, and arsenic in bacteria isolated from extreme environments in high-altitude (above 4400 m) Andean wetlands. *Current Microbiology*, *56*(5), 510–517. https://doi.org/10.1007/s00284-008-9103-2

Doit, C., Loukil, C., Simon, A., Ferroni, A., Fontan, J., Bonacorsi, S., … Bingen, E. (2004). Outbreak of *Burkholderia cepacia* Bacteremia in a Pediatric Hospital Due to Contamination of Lipid Emulsion Stoppers. *Journal of Clinical Microbiology*, *42*(5), 2227–2230. https://doi.org/10.1128/JCM.42.5.2227

Domenico, E. G. Di, Toma, L., Provot, C., Ascenzioni, F., Sperduti, I., Prignano, G., … Ensoli, F. (2016). Development of an in vitro Assay , Based on the BioFilm Ring Test ® , for Rapid Profiling of Biofilm-Growing Bacteria. *Frontiers in Microbiology*, *7*(1429), 1–14. https://doi.org/10.3389/fmicb.2016.01429

Donlan, R.M., & Costerton, J. W. (2002). Biofilms: survivalmechanisms of clinically relevant microorganisms. *Clin.Microbiol. Rev.*, *15*(2), 167–19. https://doi.org/10.1128/CMR.15.2.167

Donlan, Rodney M. (2002). Biofilms: Microbial life on surfaces. *Emerging Infectious Diseases*, *8*(9), 881–890. https://doi.org/10.3201/eid0809.020063

Douterelo, I., Sharpe, R., & Boxall, J. (2011). Influence of hydraulic regimes on bacterial community structure and composition in an experimental drinking water distribution system. *Water Research*, *47*(2), 503–516.

Dowling, A. J., Wilkinson, P. A., Holden, M. T. G., Quail, M. A., Bentley, S. D., Reger,

J., … Ffrench-Constant, R. H. (2010). Genome-wide analysis reveals loci encoding anti- macrophage factors in the human pathogen *Burkholderia pseudomallei* K96243. *PLoS ONE*, *5*(12). https://doi.org/10.1371/journal.pone.0015693

Dufour, D., Leung, V., & Lévesque, C. M. (2012). Bacterial biofilm : structure , function , and antimicrobial resistance. *Endodontic Topics*, 2–16.

Edwards, B. D., Somayaji, R., Missaghi, B., Chan, W. W., & Bois, A. J. (2017). Prosthetic joint and implant contamination caused by *Ralstonia pickettii* : a report of three cases H. *SICOT-J*, *3*(32). https://doi.org/10.1051/sicotj/2017017

Eichler, S., Christen, R., Ho, C., Westphal, P., Bo, J., Brettar, I., … Ho, M. G. (2006). Composition and Dynamics of Bacterial Communities of a Drinking Water Supply System as Assessed by RNA- and DNA-Based 16S rRNA Gene Fingerprinting. *Applied and Environmental Microbiology*, *72*(3), 1858–1872. https://doi.org/10.1128/AEM.72.3.1858

El Salabi, A., Borra, S., Toleman, M. A., Samuelsen, Ø., & Walsh, T. R. (2012). Genetic and Biochemical Characterization of a Novel Metallo-B-Lactamase, TMB-1, from an *Achromobacter xylosoxidans* Strain Isolated in Tripoli, Libya. *Antimicrobial Agents and Chemotherapy*, *56*(5), 2241–2245. https://doi.org/10.1128/AAC.05640-11

Emtiazi, F., Schwartz, T., Marten, S. M., Krolla-sidenstein, P., & Obst, U. (2004). Investigation of natural biofilms formed during the production of drinking water from surface water embankment filtration. *Water Research*, *38*, 1197–1206. https://doi.org/10.1016/j.watres.2003.10.056

Engledow, A. S., Medrano, E. G., Mahenthiralingam, E., Lipuma, J. J., & Gonzalez, C. F. (2004). Involvement of a Plasmid-Encoded Type IV Secretion System in the Plant Tissue Watersoaking Phenotype of *Burkholderia cenocepacia*. *Journal of Bacteriology*, *186*(18), 6015–6024. https://doi.org/10.1128/JB.186.18.6015

Essex-lopresti, A. E., Boddey, J. A., Thomas, R., Smith, M. P., Hartley, M. G., Atkins, T., … Titball, R. W. (2005). A Type IV Pilin , PilA , Contributes to Adherence of *Burkholderia pseudomallei* and Virulence In Vivo. *Infection and Immunity*, *73*(2), 1260–1264. https://doi.org/10.1128/IAI.73.2.1260

Estivariz, C. F., Bhatti, L. I., Jensen, B., Arduino, M., Jernigan, D., LiPuma, J., & Srinivasan, A. (2004). An Outbreak of *Burkholderia cepacia*. *CHEST*, *130*(5), 1346–1353. https://doi.org/10.1378/chest.130.5.1346

Estrela, C., Estrela, C. R., Barbin, E. L., Spano, J. C., Marchesan, M. a, & Pecora, J. D. (2002). Mechanism of action of sodium hypochlorite. *Braz Dent J*, *13*, 113–117. https://doi.org/10.1590/S0103-64402002000200007

Falcone-Dias, M. F., Centrón, D., Pavan, F., Candido, A., Moura, S., Naveca, F. G., … Farache, A. (2015). Opportunistic Pathogens and Elements of the Resistome that Are Common in Bottled Mineral Water Support the Need for Continuous Surveillance. *PLoS ONE*, 1–12. https://doi.org/10.1371/journal.pone.0121284

Falcone-Dias, M. F., Vaz-Moreira, I., & Manaia, C. M. (2012). Bottled mineral water as a potential source of antibiotic resistant bacteria. *Water Research*, *46*(11). https://doi.org/10.1016/j.watres.2012.04.007

Fekih, I. Ben, Zhang, C., Li, Y. P., & Zhao, Y. (2018). Distribution of Arsenic Resistance Genes in Prokaryotes. *Frontiers in Microbiology*, *9*(October), 1–11. https://doi.org/10.3389/fmicb.2018.02473

Fernandez, C., Wilhelmi, I., Andradas, E., Gaspar, C., Gomez, J., Romero, J., … Fereres, J. (1996). Nosocomial outbreak of *Burkholderia pickettii* infection due to a manufactured intravenous product used in three hospitals. *Clinical Infectious Diseases*, *22*(6), 1092–1095. Retrieved from http://ovidsp.ovid.com/ovidweb.cgi?T=JS&PAGE=reference&D=med4&NEWS=N&AN=8783718

Ferreira, C., Bogas, D., Bikkarolla, S. K., Varela, A. R., Linheiro, R., Nunes, O. C., … Manaia, C. M. (2019). Genetic variation in the conjugative plasmidome of a hospital effluent multidrug resistant *Escherichia coli* strain. *Chemosphere*, *220*, 748–759. https://doi.org/10.1016/j.chemosphere.2018.12.130

Ferreira Da Silva, M., Vaz-Moreira, I., Gonzalez-Pajuelo, M., Nunes, O. C., & Manaia, C. M. (2007). Antimicrobial resistance patterns in *Enterobacteriaceae* isolated from an urban wastewater treatment plant. *FEMS Microbiology Ecology*, *60*, 166–176. https://doi.org/10.1111/j.1574-6941.2006.00268.x

Ferro, P., Vaz-Moreira, I., & Manaia, C. M. (2019). Association between gentamicin resistance and stress tolerance in water isolates of *Ralstonia pickettii* and *R. mannitolilytica*. *Folia Microbiologica*, *64*(1), 63–72. https://doi.org/10.1007/s12223-018-0632-1

Fiorentino, A., Ferro, G., Alferez, M. C., Polo-Lopez, M. I., Fernandez-Ibañez, P., & Rizzo, L. (2015). Inactivation and regrowth of multidrug resistant bacteria in urban wastewater after disinfection by solar-driven and chlorination processes. *Journal of Photochemistry and Photobiology B: Biology*, *148*, 43–50. https://doi.org/10.1016/j.jphotobiol.2015.03.029

Flannagan, R. S., Aubert, D., Kooi, C., Sokol, P. A., & Valvano, M. A. (2007). *Burkholderia cenocepacia* requires a periplasmic HtrA protease for growth under thermal and osmotic stress and for survival in vivo. *Infection and Immunity*, *75*(4), 1679–1689. https://doi.org/10.1128/IAI.01581-06

Flemming, H., Wingender, J., Szewzyk, U., Steinberg, P., & Rice, S. A. (2016). Biofilms : an emergent form of bacterial life. *Nature Publishing Group*, *14*(9), 563–575. https://doi.org/10.1038/nrmicro.2016.94

França, L., Lopéz-lopéz, A., Rosselló-móra, R., & Costa, M. S. (2014). Microbial diversity and dynamics of a groundwater and a still bottled natural mineral water. *Society for Applied Microbiology and John Wiley & Sons Ltd, Environmental Microbiology*. https://doi.org/10.1111/1462-2920.12430

Gao, H., Zhang, L., Lu, Z., He, C., Li, Q., & Na, G. (2017). Complex migration of antibiotic resistance in natural aquatic environments. *Environmental Pollution*, *232*, 1–9. https://doi.org/10.1016/j.envpol.2017.08.078

Gardner, S., & Shulman, S. T. (1984). A nosocomial common source outbreak caused by *Pseudomonas pickettii*. *Pediatric Infectious Disease*, *3*(5), 420–422.

Genin, S., Brito, B., Denny, T. P., & Boucher, C. (2005). Control of the *Ralstonia solanacearum* Type III secretion system (Hrp) genes by the global virulence regulator PhcA. *FEBS Letters*, *579*(10), 2077–2081. https://doi.org/10.1016/j.febslet.2005.02.058

Genin, S., & Denny, T. P. (2012). Pathogenomics of the *Ralstonia solanacearum*

Species Complex. *Annual Review of Phytopathology*, *50*(1), 67–89. https://doi.org/10.1146/annurev-phyto-081211-173000

Gilligan, P., Lum, G., VanDamme, P., & Whittier, S. (2003). *Burkholderia, Stenotrophomonas, Ralstonia, Brevundimonas, Comamonas, Delftia, Pandoraea*, and *Acidivorax*. In A. Press (Ed.), In Manual of Clinical Microbiology, ed. Patrick R Murray, Ellen Jo Baron, James H Jorgenson, Michael A Pfaller, and Robert H Yolken (8th ed, pp. 729–748). Washington DC USA.

Gillis, M., & Logan, N. (2005a). Genus IV. *Chromobacterium* Bergonzini 1881, 153AL. In G. M. Garrity, D. J. Brenner, N. R. Krieg, & J. T. Staley (Eds.), Bergey's Manual of Systematic Bacteriology (vol 2, 2ed, pp. 824–827). New York: Springer.

Gillis, M., & Logan, N. (2005b). Genus IV. *Janthinobacterium* De Ley, Segers and Gillis 1978, 164,AL emend. Lincoln, Fermor and Tindall 1999, 1586. In G. M. Garrity, D. J. Brenner, N. R. Krieg, & J. T. Staley (Eds.), *Bergey's Manual of Systematic Bacteriology* (VOL. 2, 2e, pp. 636–642). New York: Springer.

Girlich, D., Naas, T., & Nordmann, P. (2004). OXA-60, a Chromosomal, Inducible, and Imipenem-Hydrolyzing Class D B-Lactamase from *Ralstonia pickettii. Antimicrobial Agents and Chemotherapy*, *48*(11), 4217–4225. https://doi.org/10.1128/AAC.48.11.4217

Girlich, D., Naas, T., & Nordmann, P. (2006). Regulation of class D B-lactamase gene expression in *Ralstonia pickettii*. *Microbiology*, *152*(May), 2661–2672. https://doi.org/10.1099/mic.0.29027-0

Graves, L. M., & Swaminathan, B. (2001). PulseNet standardized protocol for subtyping *Listeria monocytogenes* by macrorestriction and pulsed-field gel electrophoresis q. *International Journal of Food Microbiology*, *65*, 55–62.

Green, E. R., & Mecsas, J. (2016). Bacterial Secretion Systems – An overview. *Microbiol Spectr.*, *4*(1), 1–32.

Greub, G. (2012). Culturomics : a new approach to study the human microbiome. *Clinical Microbiology and Infection*, 7–9.

Grobner, S., Heeg, P., Autenrieth, I. B., & Schulte, B. (2007). Monoclonal outbreak of

catheter-related bacteraemia by *Ralstonia mannitolilytica* on two haemato-oncology wards. *British Infection Society*, *55*, 539–544. https://doi.org/10.1016/j.jinf.2007.07.021

Guttenplan, S. B., & Kearns, D. B. (2013). Regulation of flagellar motility during biofilm formation. *FEMS Microbiology Letters*, *37*, 849–871. https://doi.org/10.1111/1574-6976.12018

Hahn, M. W. (2004). Broad diversity of viable bacteria in ‘ sterile ’ (0 . 2 μm) filtered water. *Research in Microbiologoy*, *155*, 688–691. https://doi.org/10.1016/j.resmic.2004.05.003

Har, J. Y., Helbig, T., Lim, J. H., Fernando, S. C., Reitzel, A. M., Penn, K., & Thompson, J. R. (2015). Microbial diversity and activity in the *Nematostella vectensis* holobiont: Insights from 16S rRNA gene sequencing, isolate genomes, and a pilot-scale survey of gene expression. *Frontiers in Microbiology*, *6*(JUL). https://doi.org/10.3389/fmicb.2015.00818

Held, M. R., Begier, E. M., Beardsley, D. S., Browne, F. A., Martinello, R. A., Baltimore, R. S., … Dembry, L. (2006). Life-Threatening Sepsis Caused by *Burkholderia cepacia. Pediatrics*, *118*(1). https://doi.org/10.1542/peds.2005-2617

Hermsen, R., Deris, J. B., & Hwa, T. (2012). On the rapidity of antibiotic resistance evolution facilitated by a concentration gradient. *PNAS*, *109*(27), 10775–10780. https://doi.org/10.1073/pnas.1117716109

Hijnen, W. A. M., Beerendonk, E. F., & Medema, G. J. (2006). Inactivation credit of UV radiation for viruses, bacteria and protozoan (oo)cysts in water: A review. *Water Research*, *40*(1), 3–22. https://doi.org/10.1016/j.watres.2005.10.030

Hoefel, D., Monis, P. T., Grooby, W. L., Andrews, S., & Saint, C. P. (2005). Profiling bacterial survival through a water treatment process and subsequent distribution system. *Journal of Applied Microbiology*, *99*, 175–186. https://doi.org/10.1111/j.1365-2672.2005.02573.x

Hoffman, L. R., D’Argenio, D. A., MacCoss, M. J., Zhang, Z. Y., Jones, R. A., & Miller, S. I. (2005). Aminoglycoside antibiotics induce bacterial biofilm formation. *Nature*, *436*(7054), 1171–1175. https://doi.org/Doi 10.1038/Nature03912

Holden, M. T. G., Seth-smith, H. M. B., Crossman, L. C., Sebaihia, M., Bentley, S. D., Cerden, A. M., … Parkhill, J. (2009). The Genome of *Burkholderia cenocepacia* J2315 , an Epidemic Pathogen of Cystic Fibrosis Patients. *Journal of Bacteriology*, *191*(1), 261–277. https://doi.org/10.1128/JB.01230-08

Holden, M. T. G., Titball, R. W., Peacock, S. J., Cerdeño-, A. M., Atkins, T., Crossman, L. C., … Vesaratchaveste, M. (2004). Genomic plasticity of the causative agent of melioidosis , *Burkholderia pseudomallei*. *PNAS*, *101*(39). https://doi.org/10.1073/pnas.0403302101

Hu, Y., Zhu, Y., Ma, Y., Liu, F., Lu, N., Yang, X., … Zhu, B. (2014). Genomic insights into intrinsic and acquired drug resistance mechanisms in *Achromobacter xylosoxidans*. *Antimicrobial Agents and Chemotherapy*, *59*(2), 1152–1161. https://doi.org/10.1128/AAC.04260-14

Hugon, E., Manchandin, H., Poiree, M., Fosse, T., & Sirvent, N. (2015). *Achromobacter* bacteraemia outbreak in a paediatric onco-haematology department related to strain with high surviving ability in contaminated disinfectant atomizers. *Journal of Hospital Infection*, *89*, 116–122. https://doi.org/10.1016/j.jhin.2014.07.012

Hutchison, M. L., Poxton, I. R., & Govan, J. R. W. (1998). *Burkholderia cepacia* produces a hemolysin that is capable of inducing apoptosis and degranulation of mammalian phagocytes. *Infection and Immunity*, *66*(5), 2033–2039.

Ibrahim, M., Tang, Q., Shi, Y., Almoneafy, A., Fang, Y., Xu, L., … Xie, G. L. (2012). Diversity of potential pathogenicity and biofilm formation among *Burkholderia cepacia* complex water, clinical, and agricultural isolates in China. *World Journal of Microbiology and Biotechnology*, *28*(5), 2113–2123. https://doi.org/10.1007/s11274-012-1016-3

Inglis, T. J. J., Robertson, T., Woods, D. E., Dutton, N., & Chang, B. J. (2003). Flagellum-mediated adhesion by *Burkholderia pseudomallei* precedes invasion of *Acanthamoeba astronyxis*. *Infection and Immunity*, *71*(4), 2280–2282. https://doi.org/10.1128/IAI.71.4.2280-2282.2003

Iyobe, S., Kusadokoro, H., Takahashi, A., Yomoda, S., Okubo, T., Nakamura, A., & Hara, K. O. (2016). Detection of a Variant Metallo-B-Lactamase , IMP-10 , from Two Unrelated Strains of *Pseudomonas aeruginosa* and an *Alcaligenes*

xylosoxidans Strain. *Antimicrobial Agents and Chemotherapy*, *46*(6), 2014–2016. https://doi.org/10.1128/AAC.46.6.2014

Jakobsen, T. H., Hansen, M. A., Jensen, P. Ø., Hansen, L., Riber, L., Cockburn, A., … Bjarnsholt, T. (2013). Complete Genome Sequence of the Cystic Fibrosis Pathogen *Achromobacter xylosoxidans* NH44784-1996 Complies with Important Pathogenic Phenotypes. *PLoS ONE*, *8*(7), 8–11. https://doi.org/10.1371/journal.pone.0068484

Jia, S., Shi, P., Li, B., Zhang, T., & Zhang, X.-X. (2015). Bacterial Community Shift Drives Antibiotic Resistance Promotion during Drinking Water Chlorination. *Environmental Science and Technology*. https://doi.org/10.1021/acs.est.5b03521

Johnson, C. M., & Grossman, A. D. (2015). Integrative and Conjugative Elements (ICEs): What They Do and How They Work. *Annu Rev Genet*, *49*(13.19), 13–25. https://doi.org/10.1146/annurev-genet-112414-055018

Johnson, K. L., & Walcott, R. R. (2013). Quorum sensing contributes to seed-to-seedling transmission of *Acidovorax citrulli* on watermelon. *Journal of Phytopathology*, *161*(7–8), 562–573. https://doi.org/10.1111/jph.12106

Jones, A. M., Stanbridge, T. N., Isalska, B. J., Dodd, M. E., & Webb, A. K. (2001). *Burkholderia gladioli* : Recurrent Abscesses in a Patient with Cystic Fibrosis. *The British Infection Society*, 69–71. https://doi.org/10.1053/jinf.2000.0770

Juhas, M., Van Der Meer, J. R., Gaillard, M., Harding, R. M., Hood, D. W., & Crook, D. W. (2009). Genomic islands: Tools of bacterial horizontal gene transfer and evolution. *FEMS Microbiology Reviews*, *33*, 376–393. https://doi.org/10.1111/j.1574-6976.2008.00136.x

Jukes T H & Cantor C R. (1969). Evolution of protein molecules. *In Munro HN, Editor, Mammalian Protein Metabolism, , Academic Press, New York.*, *3*, 21–132.

Kang, Y., Liu, H., Genin, S., Schell, M. A., & Denny, T. P. (2002). *Ralstonia solanacearum* requires type 4 pili to adhere to multiple surfaces and for natural transformation and virulence. *Molecular Microbiology*, *46*(2), 427–437. https://doi.org/10.1046/j.1365-2958.2002.03187.x

Kennedy, M. P., Coakley, R. D., Donaldson, S. H., Aris, R. M., Hohneker, K., Wedd, J. P., … Yankaskas, J. R. (2007). *Burkholderia gladioli* : Five year experience in a

cystic fibrosis and lung transplantation center. *Journal of Cystic Fibrosis*, *6*, 267–273. https://doi.org/10.1016/j.jcf.2006.10.007

Khajuria, A., Praharaj, A. K., Grover, N., & Kumar, M. (2014). Emergence of VIM-2 metallo-beta-lactamase producing *Ralstonia pickettii* clinical isolate in India. *Indian Journal of Medical Microbiology*, *32*(2), 191–193. https://doi.org/10.4103/0255-0857.129831

Ko, S., Rn, H. A., Hwan, J., & Park, S. (2015). American Journal of Infection Control An outbreak of *Burkholderia cepacia* complex pseudobacteremia associated with intrinsically contaminated commercial 0.5 % chlorhexidine solution. *American Journal of Infection Control*, *43*(3), 266–268. https://doi.org/10.1016/j.ajic.2014.11.010

Koenig, D. (1997). Microbiology of the space shuttle water system. *Water Science and Technology*, *35*(11–12), 59–64. https://doi.org/10.1016/S0273-1223(97)00235-7

Kondo, M., Hirai, H., Furukawa, T., Yoshida, Y., Suzuki, A., Kawaguchi, T., & Che, F.-S. (2017). Frameshift Mutation Confers Function as Virulence Factor to Leucine-Rich Repeat Protein from *Acidovorax avenae*. *Frontiers in Plant Science*, *7*(January), 1–13. https://doi.org/10.3389/fpls.2016.01988

Kreutzer, M. F., Kage, H., Gebhardt, P., Wackler, B., Saluz, H. P., Hoffmeister, D., & Nett, M. (2011). Biosynthesis of a Complex *Yersiniabactin*-Like Natural Product via the mic Locus in Phytopathogen *Ralstonia solanacearum*. *Applied and Environmental Microbiology*, *77*(17), 6117–6124. https://doi.org/10.1128/AEM.05198-11

Krol, J. E., Wojtowicz, A. J., Rogers, L. M., Heuer, H., Smalla, K., Krone, S. M., & Top, E. M. (2013). Invasion of *E. coli* biofilms by antibiotic resistance plasmids. *Plasmid*, *70*(1), 110–119. https://doi.org/10.1016/j.plasmid.2013.03.003.Invasion

Kulakov, L. A., McAlister, M. B., Ogden, K. L., Larkin, M. J., & O'Hanlon, J. F. (2002). Analysis of bacteria contaminating ultrapure water in industrial systems. *Applied and Environmental Microbiology*, *68*(4), 1548–1555. https://doi.org/10.1128/AEM.68.4.1548-1555.2002

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7 : Molecular Evolutionary

Genetics Analysis Version 7 . 0 for Bigger Datasets Brief communication. *Molecular Biology and Evolution*, *33*(7), 1870–1874. https://doi.org/10.1093/molbev/msw054

Labarca, J. A., Trick, W. E., Peterson, C. L., Carson, L. A., Holt, S. C., Arduino, M. J., … Jarvis, W. R. (1999). A multistate nosocomial outbreak of *Ralstonia pickettii* colonization associated with an intrinsically contaminated respiratory care solution. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America*, *29*(5), 1281–1286. https://doi.org/10.1086/313458

Lacey, S., & Want, S. V. (1991). *Pseudomonas pickettii* infections in a paediatric oncology unit. *Journal of Hospital Infection*, *17*(1), 45–51. https://doi.org/10.1016/0195-6701(91)90076-K

Lagier, J., Armougom, F., Million, M., Hugon, P., Pagnier, I., Robert, C., … Gimenez, G. (2012). Microbial culturomics: paradigm shift in the human gut microbiome study. *Clinical Microbiology and Infection*, *18*(12), 1185–1193. https://doi.org/10.1111/1469-0691.12023

Landy, A., & Ross, W. (1977). Viral integration and excision: structure of the lambda att sites: DNA sequences have been determined for regions involved in lambda site-specific recombination. *Science (New York, NY)*, *197*(4309), 1147.

Lane, D. J. (1991). 16S/23S rRNA sequencing. In E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. (J. Wiley, pp. 115–175). Chichester United Kingdom.

Lautenschlager, K., Boon, N., Wang, Y., Egli, T., & Hammes, F. (2010). Overnight stagnation of drinking water in household taps induces microbial growth and changes in community composition. *Water Research*, *44*(17), 4868–4877. https://doi.org/10.1016/j.watres.2010.07.032

Lautenschlager, K., Hwang, C., Ling, F., Egli, T., Liu, W., Boon, N., … Hammes, F. (2014). ScienceDirect Abundance and composition of indigenous bacterial communities in a multi-step biofiltration-based drinking water treatment plant. *Water Research*, *62*, 40–52. https://doi.org/10.1016/j.watres.2014.05.035

Law, R. J., Hamlin, J. N. R., Sivro, A., McCorrister, S. J., Cardama, G. A., & Cardona,

S. T. (2008). A functional phenylacetic acid catabolic pathway is required for full pathogenicity of *Burkholderia cenocepacia* in the *Caenorhabditis elegans* host model. *Journal of Bacteriology*, *190*(21), 7209–7218. https://doi.org/10.1128/JB.00481-08

Lawrence, J. G. (2005). Horizontal and vertical gene transfer: the life history of pathogens. *Contributions to Microbiology*, *12*, 255–271. https://doi.org/10.1159/000081699

Leclerc, H. (2003). Relationships between common water bacteria & pathogens in drinking-water. In J. Bartram (Ed.), Heterotrophic plate counts and drinking-water safety: The significance of HPCs for water quality and the human health (84–85pp). London: IWA Publishing. Retrieved from http://www.who.int/water_sanitation_health/dwq/hpc/en/index.html

Leclerc, Henri, & Moreau, A. (2002). Microbiological safety of natural mineral water. *FEMS Microbiology Letters*, *26*, 207–222.

Leclercq, R., Cantón, R., Brown, D. F. J., Giske, C. G., Heisig, P., Macgowan, A. P., … Kahlmeter, G. (2013). EUCAST expert rules in antimicrobial susceptibility testing. *Clinical Microbiology and Infection*, *19*(2), 141–160. https://doi.org/10.1111/j.1469-0691.2011.03703.x

Lee, J., Lee, C. S., Hugunin, K. M., Maute, C. J., & Dysko, R. C. (2010). Bacteria from drinking water supply and their fate in gastrointestinal tracts of germ-free mice : A phylogenetic comparison study. *Water Research*, *44*(17), 5050–5058. https://doi.org/10.1016/j.watres.2010.07.027

Letunic, I., & Bork, P. (2016). Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees. *Nucleic Acids Research*, 1–4. https://doi.org/10.1093/nar/gkw290

Lewenza, S., Conway, B., & Greenberg, E. P. (1999). Quorum Sensing in *Burkholderia cepacia* : Identification of the LuxRI Homologs CepRI. *Journal of Bacteriology*, *181*(3), 748–756.

Lewis, K. I. M. (2001). MINIREVIEW Riddle of Biofilm Resistance. *Antimicrob Agents Chemother*, *45*(4), 999–1007. https://doi.org/10.1128/AAC.45.4.999

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., … Durbin, R. (2009). The Sequence Alignment/Map format and SAMtools. *Bioinformatics (Oxford, England)*, *25*(16), 2078–2079. https://doi.org/10.1093/bioinformatics/btp352

Li, L.-G., Xia, Y., & Zhang, T. (2016). Co-occurrence of antibiotic and metal resistance genes revealed in complete genome collection. *The ISME Journal*, 1–12. https://doi.org/10.1038/ismej.2016.155

Li, X., Hu, Y., Gong, J., Zhang, L., & Wang, G. (2013). Comparative genome characterization of *Achromobacter* members reveals potential genetic determinants facilitating the adaptation to a pathogenic lifestyle. *Applied Microbiology and Biotechnology*, *97*(14), 6413–6425. https://doi.org/10.1007/s00253-013-5018-3

Lincoln, S. P., Fermor, T. R., & Tindall, B. J. (1999). *Janthinobacterium agaricidamnosum* sp. nov., a soft rot pathogen of *Agaricus bisporus*. *International Journal of Systematic Bacteriology*, *49*(4), 1577–1589.

Lindsay, D., & von Holy, A. (2006). Bacterial biofilms within the clinical setting: what healthcare professionals should know. *Journal of Hospital Infection*, *64*(4), 313–325. https://doi.org/10.1016/j.jhin.2006.06.028

Liu, C., Yan, C., Zhang, P., Li, F., Yang, J., & Li, X. (2016). *Ralstonia mannitolilytica* - Induced Septicemia and Homology Analysis in Infected Patients : 3 Case Reports. *Jundishapur j Microbiol*, *9*(7). https://doi.org/10.5812/jjm.34373.Research

Liu, H., Kang, Y., Genin, S., Schell, M. A., & Denny, T. P. (2001). Twitching motility of *Ralstonia solanacearum* requires a type IV pilus system. *Microbiology*, *147*(2001), 3215–3229. Retrieved from http://mic.sgmjournals.org/content/journal/micro/10.1099/00221287-147-12-3215

Liu, M., Li, X., Xie, Y., Bi, D., Sun, J., Li, J., … Ou, H. (2019). ICEberg 2 . 0 : an updated database of bacterial integrative and conjugative elements. *Nucleic Acids Research*, *47*, 660–665. https://doi.org/10.1093/nar/gky1123

Loutet, S. A., & Valvano, M. A. (2010). A decade of *Burkholderia cenocepacia* virulence determinant research. *Infection and Immunity*, *78*(10), 4088–4100. https://doi.org/10.1128/IAI.00212-10

Loy, A., Beisker, W., & Meier, H. (2005). Diversity of Bacteria Growing in Natural Mineral Water after Bottling. *Applied and Environmental Microbiology*, *71*(7), 3624–3632. https://doi.org/10.1128/AEM.71.7.3624

Luk, W. K. (1996). An outbreak of pseudobacteraemia caused by *Burkholderia pickettii:* The critical role of an epidemiological link. *Journal of Hospital Infection*, *34*(1), 59–69. https://doi.org/10.1016/S0195-6701(96)90126-7

Ma, L., Li, B., Jiang, X., Wang, Y., Xia, Y., Li, A., & Zhang, T. (2017). Catalogue of antibiotic resistome and host- tracking in drinking water deciphered by a large scale survey. *Microbiome*, 1–12. https://doi.org/10.1186/s40168-017-0369-0

Madigan, M. T., Martinko Jhon, M., Bender Kelly, S., Buckley Daniel, H., & Stahl David, A. (2006). Brock Biology of Microorganisms. Fourteenth edition. Pearson, Ed, pp. 1040.

Magalhaes, M., Doherty, C., Govan, J. R. W., & Vandamme, P. (2003). Polyclonal outbreak of *Burkholderia cepacia* complex bacteraemia in haemodialysis patients. *Journal of Hospital Infection*, *6701*, 120–123. https://doi.org/10.1016/S0195-6701(03)00118-X

Magalhães, R., Almeida, G., Ferreira, V., Santos, I., Silva, J., Mendes, M. M., … Farber, J. (2015). Cheese-related listeriosis outbreak, Portugal, March 2009 to February 2012. *Euro Surviell*, 20(17), 1–6.

Mah, & O'Toole, G. A. (2001). Mechanisms of biofilm resistance to antimicrobial agents. *Trends in Microbiology*, *9*(1), 34–39. https://doi.org/10.1016/S0966-842X(00)01913-2

Maki, D. G., Klein, B. S., McCormick, R. D., Alvarado, C. J., Zilz, M. A., Stolz, S. M., … Liegel, A. R. (1991). Nosocomial *Pseudomonas pickettii* bacteremias traced to narcotic tampering. A case for selective drug screening of health care personnel. *JAMA : The Journal of the American Medical Association*, *265*(8), 981–986. https://doi.org/10.1001/jama.265.8.981

Malott, R. J., & Sokol, P. A. (2007). Expression of the bviIR and cepIR quorum-sensing systems of *Burkholderia vietnamiensis*. *Journal of Bacteriology*, *189*(8), 3006–3016. https://doi.org/10.1128/JB.01544-06

Manz, W., Wendt-Potthoff, K., Neu, T. R., Szewzyk, U., & Lawrence, J. R. (1999). Phylogenetic Composition, Spatial Structure, and Dynamics of Lotic Bacterial Biofilms Investigated by Fluorescent. *Microbial Ecology*, *37*, 225–237. https://doi.org/10.1007/s002489900148

Marchetti, R., Dillon, M. J., Burtnick, M. N., Hubbard, M. A., Kenfack, M. T., Blériot, Y., … Molinaro, A. (2015). *Burkholderia pseudomallei* Capsular Polysaccharide Recognition by a Monoclonal Antibody Reveals Key Details toward a Biodefense Vaccine and Diagnostics against Melioidosis. *ACS Chemical Biology*, *10*(10), 2295–2302. https://doi.org/10.1021/acschembio.5b00502

Maroye, P., Doermann, H. P., Rogues, A. M., Gachie, J. P., & Mégraud, F. (2000). Investigation of an outbreak of *Ralstonia pickettii* in a paediatric hospital by RAPD. *Journal of Hospital Infection*, *44*(4), 267–272. https://doi.org/10.1053/jhin.1999.0691

Marsalek, J., Jimenez-Cisneros, B., Malmquist, M., Goldenfum, J., & Chocat, B. (2006). Urban water cycle processes and interactions. Paris: UNESCO/IHP.

McDonnell, G., & Russell, D. (1999). Antiseptics and Disinfectants : Activity , Action , and Resistance. *Clinical Microbiology Reviews*, *12*(1), 147–179. https://doi.org/0893-8512/99/$04.00+0

Mckinney, R. E. (1953). Staining bacterial polysaccharides. *J Bacteriology*, *66*, 453–454.

McNeil, M. M., Solomon, S. L., Anderson, R. L., Davis, B. J., Spengler, R. F., Reisberg, B. E., … Martone, W. J. (1985). Nosocomial *Pseudomonas pickettii* colonization associated with a contaminated respiratory therapy solution in a special care nursery. *Journal of Clinical Microbiology*, *22*(6), 903–907.

Meng, F., Babujee, L., Jacobs, J. M., & Allen, C. (2015). Comparative transcriptome analysis reveals cool virulence factors of *Ralstonia solanacearum* race 3 biovar 2. *PLoS ONE*, *10*(10), 1–22. https://doi.org/10.1371/journal.pone.0139090

Messi, P., Guerrieri, E., & Bondi, M. (2005). Antibiotic resistance and antibacterial activity in heterotrophic bacteria of mineral water origin. *Science of The Total Environment*, *346*, 213–219.

Mi, Z., Dai, Y., Xie, S., Chen, C., & Zhang, X. (2015). ScienceDirect Impact of disinfection on drinking water biofilm bacterial community. *Journal of Environmental Sciences*, *37*, 200–205.

Mijnendonckx, K., Provoost, A., & Ott, C. M. (2013). Characterization of the Survival Ability of *Cupriavidus metalliduran*s and *Ralstonia pickettii* from Space-Related Environments. *Microbiology Ecology*, *63*, 347–360. https://doi.org/10.1007/s00248-012-0139-2

Mima, T., & Schweizer, H. P. (2010). The BpeAB-OprB Efflux Pump of *Burkholderia pseudomallei* 1026b Does Not Play a Role in Quorum Sensing , Virulence Factor Production , or Extrusion of Aminoglycosides but Is a Broad-Spectrum Drug Efflux System. *Antimicrobial Agents and Chemotherapy*, *54*(8), 3113–3120. https://doi.org/10.1128/AAC.01803-09

Muangman, S., Korbsrisate, S., Muangsombut, V., & Srinon, V. (2011). BopC is a type III secreted effector protein of *Burkholderia pseudomallei*. *FEMS Microbiology Letters*, *323*(2009), 75–82. https://doi.org/10.1111/j.1574-6968.2011.02359.x

Munita, J. M., & Arias, C. A. (2016). Mechanisms of Antibiotic Resistance. *Microbiol Spectr.*, *4*(2), 1–37. https://doi.org/10.1128/microbiolspec.VMBF-0016-2015.

Narciso-Da-Rocha, C., Vaz-Moreira, I., Svensson-Stadler, L., Moore, E. R. B., & Manaia, C. M. (2013). Diversity and antibiotic resistance of *Acinetobacter* spp. in water from the source to the tap. *Applied Microbiology and Biotechnology*, *97*, 329–340. https://doi.org/10.1007/s00253-012-4190-1

Nasser, R. M., Rahi, A., Daoud, Z., Hakime, N., & Almawi, W. (2004). Outbreak of *Burkholderia cepacia* Bacteremia Traced to Contaminated Hospital Water Used for Dilution of an Alcohol Skin Antiseptic. *Infection Control*, (April). https://doi.org/10.1086/502384

Neuwirth, C., Freby, C., Ogier-desserrey, A., Perez-martin, S., & Houzel, A. (2006). VEB-1 in *Achromobacter xylosoxidans* from Cystic Fibrosis. *Emerging Infectious Diseases*, *12*(11), 1737–1739.

Nikaido, H., & Takatsuka, Y. (2009). Mechanisms of RND multidrug efflux pumps. *Biochimica et Biophysica Acta*, *1794*, 769–781.

https://doi.org/10.1016/j.bbapap.2008.10.004

Nordmann, P., Poirel, L., Kubina, M., Casetta, A., & Naas, T. (2000). Biochemical-Genetic Characterization and Distribution of OXA-22 , a Chromosomal and Inducible Class D B -Lactamase from *Ralstonia* (*Pseudomonas) pickettii*. *Antimicrobial Agents and Chemotherapy*, *44*(8), 2201–2204.

Norton, C. D., & LeChevallier, M. W. L. E. (2000). A Pilot Study of Bacteriological Population Changes through Potable Water Treatment and Distribution. *Applied and Environmental Microbiology*, *66*(1), 268–276.

O'Toole, G. A. O., & Kolter, R. (1998). Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Molecular Microbiology*, *30*(2), 295–304.

Orme, J., Rivera-Bonilla, T., Loli, A., & Blattman, N. N. (2015). Native valve endocarditis due to *Ralstonia pickettii*: A case report and literature review. *Case Reports in Infectious Diseases*, *[published*, 1-9. doi: 10.1155/2015/324675.

Palleroni, N. J. (2005). Genus I. *Burkholderia* De Yabuuchi, Kosako, Oyaizu, Hotta, Hashimoto, Ezaki and Arakawa 1993, 398VP (Effective publication: Yabuuchi, Kosako, Oyaizu Yano, Hotta, Hashimoto, Ezaki and Arakawa 1992,1268) emend. Gillis, Van, Bardin, Goor, Hebbar, Willens, Seg. In G. M. Garrity, D. J. Brenner, N. R. Krieg, & J. T. Staley (Eds.), Bergey's Manual of Systematic Bacteriology (vol 2, 2ed, pp. 575–599). New York: Springer.

Pan, W., Zhao, Z., & Dong, M. (2011). Lobar pneumonia caused by *Ralstonia pickettii* in a sixty-five-year-old Han Chinese man: a case report. *Journal of Medical Case Reports*, *5*(1), 377. https://doi.org/10.1186/1752-1947-5-377

Parte, A. C. (2019). LPSN – List of Prokaryotic names with Standing in Nomenclature (bacterio . net), 20 years on. *International Journal of Systematic and Evolutionary Microbiology*, 1825–1829. https://doi.org/10.1099/ijsem.0.002786

Paterson, J., & Gross, H. (2018). Draft Genome Sequence and Annotation of the Phytopathogenic *Ralstonia piclettii* (Previously *Burkholderia glumae*) Strain ICMP-8657. *American Society for Microbiology*, 4–6.

Patijanasoontorn, B., Wilailackana, C., Noppawinyoowong, C., Boonma, P.,

Sitthikesorn, J., Chetchotisakd, P., & Simajareuk, K. (1992). Hospital acquired *Janthinobacterium lividum* septicemia in Srinagarind Hospital. *Journal of the Medical Association of Thailland*, *75*(2).

Paul, D., Poddar, S., & Sar, P. (2014). Characterization of arsenite-oxidizing bacteria isolated from arsenic-contaminated groundwater of West Bengal. *Journal of Environmental Science and Health, Part A*, *49*(October), 1481–1492. https://doi.org/10.1080/10934529.2014.937162

Pepper, I. L., Rusin, P., Quintanar, D. R., Haney, C., Josephson, K. L., & Gerba, C. P. (2004). Tracking the concentration of heterotrophic plate count bacteria from the source to the consumer's tap. *International Journal of Food Microbiology*, *92*, 289–295. https://doi.org/10.1016/j.ijfoodmicro.2003.08.021

Perry, J. A., Westman, E. L., & Wright, G. D. (2014). ScienceDirect The antibiotic resistome : what's new ? *Current Opinion in Microbiology*, *21*, 45–50. https://doi.org/10.1016/j.mib.2014.09.002

Persson, O. P., Pinhassi, J., Riemann, L., Marklund, B., Rhen, M., Normark, S., … Hagström, Å. (2009). High abundance of virulence gene homologues in marine bacteria. *Environmental Microbiology*, *11*(6), 1348–1357. https://doi.org/10.1111/j.1462-2920.2008.01861.x

Pierro, F. Di. (2015). Antibiotic resistance. A role for nutraceuticals? Nutrafoods. Springer. https://doi.org/10.1007/s13749-015-0015-z

Pinto, A. J., Xi, C., & Raskin, L. (2012). Bacterial Community Structure in the Drinking Water Microbiome Is Governed by Filtration Processes. *Environmental Science and Technology*, *46*, 8851–8859.

Pitt, T. L., Kaufmann, M. E., Patel, F. S., Benget, L. C. A., Gaskin, S., & Livermore, D. M. (1996). Type characterisation and antibiotic susceptibility of *Burkholderia (Pseudomonasl cepacia* isolates from patients with cystic fibrosis in the United Kingdom and the Republic of Ireland. *J. Med Microbiol*, *44*(1996), 203–210.

Poitelon, J.-B., Joyeux, M., Welte, B., Duguet, J., Prestel, E., Lespinet, O., & Dubow, M. S. (2009). Assessment of phylogenetic diversity of bacterial microflora in drinking water using serial analysis of ribosomal sequence tags. *Water Research*,

43, 4197–4206. https://doi.org/10.1016/j.watres.2009.07.020

Pothula, K. R., Solano, C. J. F., & Kleinekathöfer, U. (2016). Biochimica et Biophysica Acta Simulations of outer membrane channels and their permeability ☆. *BBA - Biomembranes*, *1858*(7), 1760–1771. https://doi.org/10.1016/j.bbamem.2015.12.020

Poueymiro, M., & Genin, S. (2009). Secreted proteins from *Ralstonia solanacearum*: a hundred tricks to kill a plant. *Current Opinion in Microbiology*, *12*(1), 44–52. https://doi.org/10.1016/j.mib.2008.11.008

Puhar, A., & Sansonetti, P. J. (2014). Type III secretion system. *Current Biology*, *24*(17), R784–R791. https://doi.org/10.1016/j.cub.2014.07.016

Ramoa, A. R., Elena, T., Rodrigo, P. de O., Di Vittorio, F., Montanari, A., & Monteiro, A. (2015). Review of global change pressures on Urban Water Cycle Systems. Assessment of TRUST Pilots. EUROPEAN UNION: TRUST Pilots.

Rang, C., Alix, E., Felix, C., Heitz, A., & Tasse, L. (2006). Dual role of the MgtC virulence factor in host and non-host environments. *Molecular Microbiology*, *63*(2), 605–622. https://doi.org/10.1111/j.1365-2958.2006.05542.x

Reckseidler-zenteno, S. L., Devinney, R., & Woods, D. E. (2005). The Capsular Polysaccharide of *Burkholderia pseudomallei* Contributes to Survival in Serum by Reducing Complement Factor C3b Deposition. *Infection and Immunity*, *73*(2), 1106–1115. https://doi.org/10.1128/IAI.73.2.1106

Revetta, R. P., Pemberton, A., Lamendella, R., Iker, B., & Santo, J. W. (2010). Identification of bacterial populations in drinking water using 16S rRNA-based sequence analyses. *Water Research*, *44*(5), 1353–1360. https://doi.org/10.1016/j.watres.2009.11.008

Rhodes, K. A., & Schweizer, H. P. (2016). Antibiotic resistance in *Burkholderia* species. *Drug Resistance Updates*, *28*, 82–90. https://doi.org/10.1016/j.drup.2016.07.003

Riccio, M. L., Pallecchi, L., Fontana, R., & Rossolini, G. M. (2001). In70 of Plasmid pAX22 , a bla VIM-1 -Containing Integron Carrying a New Aminoglycoside Phosphotransferase Gene Cassette. *Antimicrobial Agents and Chemotherapy*,

45(4), 1249–1253. https://doi.org/10.1128/AAC.45.4.1249

Riley, P. S., & Weaver, R. E. (1975). Recognition of *Pseudomonas pickettii* in the clinical laboratory: biochemical characterization of 62 strains. *Journal of Clinical Microbiology*, *1*(1), 61–64.

Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M. C., … Fatta-Kassinos, D. (2013). Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review. *Science of the Total Environment*, *447*, 345–360. https://doi.org/10.1016/j.scitotenv.2013.01.032

Roberts, L. A., Collignon, P. J., Cramp, V. B., Alexander, S., McFarlane, A. E., Graham, E., … Hellyar, A. (1990). An Australia-wide epidemic of *Pseudomonas pickettii* bacteraemia due to contaminated "sterile" water for injection. *Medical Journal of Australia*, *152*(12), 652–655.

Rode, T. M., Langsrud, S., Holck, A., & Møretrø, T. (2007). Different patterns of biofilm formation in *Staphylococcus aureus* under food-related stress conditions. *International Journal of Food Microbiology*, *116*, 372–383. https://doi.org/10.1016/j.ijfoodmicro.2007.02.017

Rutherford, S. T., & Bassler, B. L. (2012). Bacterial Quorum Sensing : Its Role in Virulence and Possibilities for Its Control. *Perspectives in Medicine*, 1–26.

Ryan, M. P., Pembroke, J. T., & Adley, C. C. (2007). *Ralstonia pickettii* in environmental biotechnology: Potential and applications. *Journal of Applied Microbiology*, *103*, 754–764. https://doi.org/10.1111/j.1365-2672.2007.03361.x

Ryan, M, & Adley, C. C. (2013). The antibiotic susceptibility of water-based bacteria *Ralstonia pickettii* and *Ralstonia insidiosa*. *Journal of Medical Microbiology*, *62*, 1025–1031. https://doi.org/10.1099/jmm.0.054759-0

Ryan, M P, & Adley, C. C. (2014). *Ralstonia* spp .: emerging global opportunistic pathogens. *European Journal of Clinical Microbiology and Infectious Diseases*, *33*(3), 291–304. https://doi.org/10.1007/s10096-013-1975-9

Ryan, M, Pembroke, J., & Adley, C. (2006). *Ralstonia pickettii*: a persistent Gram-negative nosocomial infectious organism. *Journal of Hospital Infection*, *62*, 278–284. https://doi.org/10.1016/j.jhin.2005.08.015

Ryan, Michael P, Pembroke, J. T., & Adley, C. C. (2009). Novel Tn4371-ICE like element in *Ralstonia pickettii* and genome mining for comparative elements. *BMC Microbiology*, *9*, 242. https://doi.org/10.1186/1471-2180-9-242

Ryan, Michael P, Pembroke, J. T., & Adley, C. C. (2011). Genotypic and phenotypic diversity of *Ralstonia pickettii* and *Ralstonia insidiosa* isolates from clinical and environmental sources including High-purity Water. Diversity in Ralstonia pickettii. *BMC Microbiology*, *11*(1), 194. https://doi.org/10.1186/1471-2180-11-194

Ryan, MP. (2009). Genotypic and Phenotypic Analysis of *Ralstonia pickettii* High Purity Water Isolates (PhD thesis). Limerick, Ireland: University of Limerick, Ireland.

Salyers, A. A., Shoemaker, N. B., Stevens, A. M., & Li, L. Y. (1995). Conjugative transposons: an unusual and diverse set of integrated gene transfer elements. *Microbiological Reviews*, *59*(4), 579–590.

Schaefers, M., Boisvert, N. & Priebe, G. (2017). Sequence variations in the *Burkholderia dolosa* two-component system fixLJ modulate phospho-transfer and virulence, pp. S356-S356, Wiley 111 River st, Hoboken 07030-5774, NJ USA.

Schell, M. A. (2000). Control of virulence and pathogenecity genes of *Ralstonia solanacearum* by an elaborate sensory network. *Annual Review of Phytopathology*, *38*, 263–292.

Schell, M. A., Lipscomb, L., & DeShazer, D. (2008). Comparative genomics and an insect model rapidly identify novel virulence genes of *Burkholderia mallei*. *Journal of Bacteriology*, *190*(7), 2306–2313. https://doi.org/10.1128/JB.01735-07

Schmidt, M. A., Balsanelli, E., Faoro, H., Cruz, L. M., Wassem, R., De Baura, V. A., … Monteiro, R. A. (2012). The type III secretion system is necessary for the development of a pathogenic and endophytic interaction between *Herbaspirillum rubrisubalbicans* and *Poaceae*. *BMC Microbiology*, *12*. https://doi.org/10.1186/1471-2180-12-98

Schwarz, S., West, T. E., Boyer, F., Chiang, W. C., Carl, M. A., Hood, R. D., … Mougous, J. D. (2010). *Burkholderia* type vi secretion systems have distinct roles

in eukaryotic and bacterial cell interactions. *PLoS Pathogens*, *6*(8), 77–78. https://doi.org/10.1371/journal.ppat.1001068

Schwering, M., Song, J., Louie, M., Turner, R. J., & Ceri, H. (2013). Biofouling : The Journal of Bioadhesion and Biofilm Multi-species biofilms defined from drinking water microorganisms provide increased protection against chlorine disinfection. *Biofouling*, 37–41. https://doi.org/10.1080/08927014.2013.816298

Seiler, C., & Berendonk, T. U. (2012). Heavy metal driven co-selection of antibiotic resistance in soil and water bodies impacted by agriculture and aquaculture. *Frontiers in Microbiology*, *3*(December), 1–10. https://doi.org/10.3389/fmicb.2012.00399

Seob, S. K., Han, K., Lee, J., Bok, S., Ra, B., Jin, S., … Sik, H. (2005). Imipenem-resistant *Achromobacter xylosoxidans* carrying bla VIM-2 -containing class 1 integron. *Diagnostic Microbiology and Infectious Disease*, *53*, 215–220. https://doi.org/10.1016/j.diagmicrobio.2005.06.018

Seong Lim, C. T., & Lee, S. E. (2017). A rare case of *Ralstonia mannitolilytica* infection in an end stage renal patient on maintenance dialysis during municipal water contamination. *Pak J Med Sci*, *33*(4), 1047–1049.

Sharma, D., Sharma, P., Soni, P., & Gupta, B. (2017). *Ralstonia picketti* neonatal sepsis : a case report. *BMC Research Notes*, 1–4. https://doi.org/10.1186/s13104-016-2347-1

Simões, L. C., Simões, M., & Vieira, M. J. (2007). Biofilm interactions between distinct bacterial genera isolated from drinking water. *Applied and Environmental Microbiology*, *73*(19), 6192–6200. https://doi.org/10.1128/AEM.00837-07

Sirinavin, S., Techasaensiri, C., Benjaponpitak, S., Pornkul, R., & Vorachit, M. (2005). Invasive *Chromobacterium violaceum* infection in children: Case report and review. *The Pediatric Infectious Disease Journal*, *24*(6), 13–15. https://doi.org/10.1097/01.inf.0000164761.81491.3f

Sitthidet, C., Korbsrisate, S., Layton, A. N., Field, T. R., Stevens, M. P., & Stevens, J. M. (2011). Identification of Motifs of *Burkholderia pseudomallei* BimA Required. *Journal of Bacteriology*, *193*(8), 1901–1910. https://doi.org/10.1128/JB.01455-10

Sitthidet, C., Stevens, J. M., Field, T. R., Layton, A. N., Korbsrisate, S., & Stevens, M. P. (2010). Actin-Based Motility of *Burkholderia thailandensis* Requires a Central Acidic Domain of BimA That Recruits and Activates the Cellular. *Journal of Bacteriology*, *192*(19), 5249–5252. https://doi.org/10.1128/JB.00608-10

Sokol, P. A., Darling, P., Woods, D. E., & Mahenthiralingam, E. (1999). Role of Ornibactin Biosynthesis in the Virulence of *Burkholderia cepacia* : Characterization of pvdA , the Gene Encoding L -Ornithine N 5 -Oxygenase. *Infection and Immunity*, *67*(9), 4443–4455.

Song, Y., Xie, C., Ong, Y., Gan, Y., & Chua, K. (2005). The BpsIR Quorum-Sensing System of *Burkholderia pseudomallei*. *Journal of Bacteriology*, *187*(2), 785–790. https://doi.org/10.1128/JB.187.2.785

Soto, S. M. (2013). Role of efflux pumps in the antibiotic resistance of bacteria embedded in a biofilm Role of efflux pumps in the antibiotic resistance of bacteria embedded in a biofilm. *Virulence*, *5594*. https://doi.org/10.4161/viru.23724

Spirig, T., Tiaden, A., Kiefer, P., Buchrieser, C., Vorholt, J. A., & Hilbi, H. (2008). The *Legionella autoinducer* synthase LqsA produces an α-hydroxyketone signaling molecule. *Journal of Biological Chemistry*, *283*(26), 18113–18123. https://doi.org/10.1074/jbc.M801929200

Srinivasan, S., Harrington, G. W., Xagoraraki, I., & Goel, R. (2008). Factors affecting bulk to total bacteria ratio in drinking water distribution systems. *Water Research*, *42*, 3393–3404. https://doi.org/10.1016/j.watres.2008.04.025

Stein, R. A. (2011). Antibiotic Resistance : A Global , Interdisciplinary Concern The Emergence of Resistant Bacteria. *The American Biology Teacher*, *73*(6), 314–321. https://doi.org/10.1525/abt.2011.73.6.3

Steindler, L., & Venturi, V. (2007). Detection of quorum-sensing N-acyl homoserine lactone signal molecules by bacterial biosensors. *FEMS Microbiology Letters*, *266*(1), 1–9. https://doi.org/10.1111/j.1574-6968.2006.00501.x

Stelzmueller, I., Biebl, M., Wiesmayr, S., Eller, M., Hoeller, E., Fille, M., … Bonatti, H. (2006). *Ralstonia pickettii* - Innocent by bystander or a potential threat? *Clinical Microbiology and Infection*, *12*(1), 99–101. https://doi.org/10.1111/j.1469-

0691.2005.01309.x

Stepanauskas, R., Glenn, T. C., Jagoe, C. H., Tuckfield, R. C., Lindell, A. H., King, C. J., & McArthur, J. V. (2006). Coselection for microbial resistance to metals and antibiotics in freshwater microcosms. *Environmental Microbiology*, *8*(9), 1510–1514. https://doi.org/10.1111/j.1462-2920.2006.01091.x

Stevens, M. P., Friebel, A., Taylor, L. A., Wood, M. W., Brown, P. J., Hardt, W., & Galyov, E. E. (2003). A *Burkholderia pseudomallei* Type III Secreted Protein , BopE, Facilitates Bacterial Invasion of Epithelial Cells and Exhibits Guanine Nucleotide Exchange Factor Activity. *Journal of Bacteriology*, *185*(16), 4992–4996. https://doi.org/10.1128/JB.185.16.4992

Stevens, M. P., Stevens, J. M., Jeng, R. L., Taylor, L. A., Wood, M. W., Hawes, P., … Galyov, E. E. (2005). Identification of a bacterial factor required for actin-based motility of *Burkholderia pseudomallei*. *Molecular Microbiology*, *56*(1), 40–53. https://doi.org/10.1111/j.1365-2958.2005.04528.x

Stevens, M. P., Wood, M. W., Taylor, L. A., Monaghan, P., Hawes, P., Jones, P. W., … Galyov, E. E. (2002). An Inv / Mxi-Spa-like type III protein secretion system in *Burkholderia pseudomallei* modulates intracellular behaviour of the pathogen. *Molecular Microbiology*, *46*(3), 649–659.

Stone, J. K., DeShazer, D., Brett, P. J., & Burtnick, M. N. (2014). Melioidosis: Molecular aspects of pathogenesis. *Expert Review of Anti-Infective Therapy*, *12*(12), 1487–1499. https://doi.org/10.1586/14787210.2014.970634

Subramoni, S., & Sokol, P. A. (2012). Virulence. *Review Future Microbiology*, *7*(12), 1373–1387.

Subsin, B., Chambers, C. E., Visser, M. B., & Sokol, P. A. (2007). Identification of genes regulated by the cepIR quorum-sensing system in *Burkholderia cenocepacia* by high-throughput screening of a random promoter library. *Journal of Bacteriology*, *189*(3), 968–979. https://doi.org/10.1128/JB.01201-06

SWITCH. (2010). Approach to Strategic planning for Integrated Urban Water Management *(IUWM)*. SWITCH-IUWM.UE.

Taboada, B., Estrada, K., Ciria, R., & Merino, E. (2018). Genome analysis Operon-

mapper : a web server for precise operon identification in bacterial and archaeal genomes. *Bioinformatics*, *34*(23), 4118–4120. https://doi.org/10.1093/bioinformatics/bty496

Tamames, J., Abellán, J. J., Pignatelli, M., Camacho, A., & Moya, A. (2010). Environmental distribution of prokaryotic taxa. *BMC Microbiology*, *10*, 85. https://doi.org/10.1186/1471-2180-10-85

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6 : Molecular Evolutionary Genetics Analysis Version 6 . 0. *Molecular Biology and Evolution*, *30*(12), 2725–2729. https://doi.org/10.1093/molbev/mst197

Tan, K. S., Chen, Y., Lim, Y., Tan, G., Liu, Y., Lim, Y., … Gan, Y. (2010). Suppression of Host Innate Immune Response by *Burkholderia pseudomallei* through the Virulence Factor TssM. *The Journal of Immunology*, *184*, 5160–5171. https://doi.org/10.4049/jimmunol.0902663

Tena, D., Carranza, R., Barbera, J. R., Valdezate, S., Garrancho, J. M., Arranz, M., & Saez-Nieto, J. A. (2005). Outbreak of long-term intravascular catheter-related bacteremia due to *Achromobacter xylosoxidans* subspecies *xylosoxidans* in a hemodialysis unit. *Euro J Clin Microbiol Infect Dis*, 727–732. https://doi.org/10.1007/s10096-005-0028-4

Thibault, F. M., Hernandez, E., Vidal, D. R., Girardet, M., & Cavallo, J. D. (2004). Antibiotic susceptibility of 65 isolates of *Burkholderia pseudomallei* and *Burkholderia mallei* to 35 antimicrobial agents. *Journal of Antimicrobial Chemotherapy*, *54*(6), 1134–1138. https://doi.org/10.1093/jac/dkh471

Thomas, V., Mcdonnell, G., Denyer, S. P., & Maillard, J. (2010). Free-living amoebaeand their intracellular pathogenic microorganisms: risks for water quality. *FEMS Microbiol Rev*, *34*, 231–259. https://doi.org/10.1111/j.1574-6976.2009.00190.x

Thomson, E. L. S., & Dennis, J. J. (2012). A *Burkholderia cepacia* complex non-ribosomal peptide-synthesized toxin is hemolytic and required for full virulence A. *Virulence*, *5594*(May). https://doi.org/10.4161/viru.19355

Tian, Y., Zhao, Y., Wu, X., Liu, F., Hu, B., & Walcott, R. R. (2015). The type VI

protein secretion system contributes to biofilm formation and seed-to-seedling transmission of *Acidovorax citrulli* on melon. *Molecular Plant Pathology*, *16*(1), 38–47. https://doi.org/10.1111/mpp.12159

Turgutalp, K., Kiykim, A., Ersoz, G., & Kaya, A. (2012). Fatal catheter-related bacteremia due to *Alcaligenes (Achromobacter) xylosoxidans* in a hemodialysis patient. *Int Urol Nephrol*, *44*, 1281–1283. https://doi.org/10.1007/s11255-011-0003-1

Udayappan, S. D., Kovatcheva-datchary, P., Bakker, G. J., Havik, R., Herrema, H., Cani, P. D., … Vos, W. M. De. (2017). Intestinal *Ralstonia pickettii* augments glucose intolerance in obesity. *PLoS ONE*, 1–15.

Ulrich, R. L., Deshazer, D., Brueggemann, E. E., Hines, H. B., Oyston, P. C., Jeddeloh, J. A., & Ulrich, R. L. (2004). Role of quorum sensing in the pathogenicity of *Burkholderia pseudomallei*. *Journal of Medical Microbiology*, *53*(53), 1053–1064. https://doi.org/10.1099/jmm.0.45661-0

Urban, T. A., Griffith, A., Torok, A. M., Smolkin, M. E., Burns, J. L., & Goldberg, J. B. (2004). Contribution of *Burkholderia cenocepacia* Flagella to Infectivity and Inflammation. *Infection and Immunity*, *72*(9), 5126–5134. https://doi.org/10.1128/IAI.72.9.5126

Valade, E., Thibault, F. M., Gauthier, Y. P., Palencia, M., Popoff, M. Y., Vidal, D. R., & Pseudomallei, P. Q. S. I. N. B. (2004). The PmlI-PmlR Quorum-Sensing System in *Burkholderia pseudomallei* Plays a Key Role in Virulence and Modulates Production of the MprA Protease. *Journal of Applied Microbiology*, *186*(8), 2288–2294. https://doi.org/10.1128/JB.186.8.2288

Valls, M., Genin, S., & Boucher, C. (2006). Integrated regulation of the type III secretion system and other virulence determinants in *Ralstonia solanacearum*. *PLoS Pathogens*, *2*(8), 0798–0807. https://doi.org/10.1371/journal.ppat.0020082

Vander Broek, C. W., & Stevens, J. M. (2017). Type III Secretion in the Melioidosis Pathogen *Burkholderia pseudomallei*. *Frontiers in Cellular and Infection Microbiology*, *7*(June). https://doi.org/10.3389/fcimb.2017.00255

Vaneechoutte, M., De Baere, T., Wauters, G., Steyaert, S., Claeys, G., Vogelaers, D., &

Verschraegen, G. (2001). One case each of recurrent meningitis and hemoperitoneum infection with *Ralstonia mannitolilytica*. *Journal of Clinical Microbiology*, *39*(12), 4588–4590. https://doi.org/10.1128/JCM.39.12.4588-4590.2001

Vaneechoutte, Mario, Kämpfer, P., De Baere, T., Falsen, E., & Verschraegen, G. (2004). Wautersia gen. nov., a novel genus accomodating the phylogenetic lineage including *Ralstonia eutropha* and related species, and proposal of *Ralstonia [Pseudomonas] syzygii* (Roberts et al. 1990) comb. nov. *International Journal of Systematic and Evolutionary Microbiology*, *54*, 317–327. https://doi.org/10.1099/ijs.0.02754-0

Vaz-Moreira, I., Nunes, O. C., & Manaia, C. M. (2017). Ubiquitous and persistent *Proteobacteria* and other Gram-negative bacteria in drinking water. *Science of the Total Environment*, *586*. https://doi.org/10.1016/j.scitotenv.2017.02.104

Vaz-Moreira, I., Egas, C., Nunes, O. C., & Manaia, C. M. (2013). Bacterial diversity from the source to the tap: A comparative study based on 16S rRNA gene-DGGE and culture-dependent methods. *FEMS Microbiology Ecology*, *83*, 361–374. https://doi.org/10.1111/1574-6941.12002

Vaz-Moreira, I., Nunes, O. C., & Manaia, C. M. (2011). Diversity and antibiotic resistance patterns of *Sphingomonadaceae* isolates from drinking water. *Applied and Environmental Microbiology*, *77*(16), 5697–5706. https://doi.org/10.1128/AEM.00579-11

Vaz-Moreira, I., Nunes, O. C., & Manaia, C. M. (2012). Diversity and antibiotic resistance in *Pseudomonas* spp. from drinking water. *Science of the Total Environment*, *426*, 366–374. https://doi.org/10.1016/j.scitotenv.2012.03.046

Vaz-Moreira, I., Nunes, O. C., & Manaia, C. M. (2014). Bacterial diversity and antibiotic resistance in water habitats: Searching the links with the human microbiome. *FEMS Microbiology Reviews*, *38*, 761–778. https://doi.org/10.1111/1574-6976.12062

Vaz-Moreira, I., Tamames, J., Martínez, J. L., & Manaia, C. M. (2016). Draft Genome Sequences of Two *Ralstonia pickettii* Strains with Different Aminoglycoside Resistance Phenotypes. *Genome Announcements*, *4*(6), e01257-16.

https://doi.org/10.1128/genomeA.01257-16

Verschraegen, G., Claeys, G., Meeus, G., & Delanghe, M. (1985). *Pseudomonas pickettii* as a cause of pseudobacteremia. *Journal of Clinical Microbiology*, *21*(2), 278–279.

Vincenti, S., Quaranta, G., De Meo, C., Bruno, S., Ficarra, M. G., Carovillano, S., … Laurenti, P. (2014). Non-fermentative gram-negative bacteria in hospital tap water and water used for haemodialysis and bronchoscope flushing: Prevalence and distribution of antibiotic resistant strains. *Science of the Total Environment*, *499*, 47–54. https://doi.org/10.1016/j.scitotenv.2014.08.041

Vu-Thien, H., Darbord, J. C., Moissenet, D., Dulot, C., Dufourcq, J. B., Marsol, P., & Garbarg-Chenon. A. (1998). Investigation of an Outbreak of Wound Infections due to *Alcaligenes xylosoxidans* Transmitted by Chlorhexidine in a Burns Unit. *Euro J Clin Microbiol Infect Dis*, 724–726.

Wang, A. F., Qiao, M., & Chen, Z. (2015). Antibiotic resistance genes in manure-amended soil and vegetables at harvest. *Journal of Hazardous Materials*, 1–48. https://doi.org/10.1016/j.jhazmat.2015.05.028

Wang, T., Guan, W., Huang, Q., Yang, Y., Yan, W., Sun, B., & Zhao, T. (2016). Quorum-sensing contributes to virulence, twitching motility, seed attachment and biofilm formation in the wild type strain Aac-5 of *Acidovorax citrulli*. *Microbial Pathogenesis*, *100*(September 2016), 133–140. https://doi.org/10.1016/j.micpath.2016.08.039

Waugh, J. B., Granger, W. M., & Gaggar, A. (2010). Incidence, Relevance and Response for *Ralstonia* Respiratory Infections. *Clin Lab Sci*, *23*(2), 99–106. https://doi.org/10.1016/j.immuni.2010.12.017.Two-stage

Weber, D. J., Rutala, W. A., & Sickbert-bennett, E. E. (2007). Outbreaks Associated with Contaminated Antiseptics and Disinfectants. *Antimicrobial Agents and Chemotherapy*, *51*(12), 4217–4224. https://doi.org/10.1128/AAC.00138-07

White, P. A., Iver, C. J. M. C., & Rawlinson, W. D. (2001). Integrons and Gene Cassettes in the *Enterobacteriaceae*. *Antimicrobial Agents and Chemotherapy*, *45*(9), 2658–2661. https://doi.org/10.1128/AAC.45.9.2658

WHO. (2011). Guidelines for Drinking-water Quality. (W. World Health Organization, Ed.). Geneve: WHO library.

WHO. (2012). Pharmaceuticals in drinking-water. Geneve: WHO library.

Wikraiphat, C., Charoensap, J., Utaisincharoen, P., & Wongratanacheewin, S. (2009). Comparative in vivo and in vitro analyses of putative virulence factors of *Burkholderia pseudomallei* using lipopolysaccharide, capsule and flagellin mutants. *FEMS Immunology and Medical Microbiology*, *56*, 253–259. https://doi.org/10.1111/j.1574-695X.2009.00574.x

Willens, A., Mergaert, J., & Swings, J. (2005). Genus X. Variovorax Willems, De Ley, Gillis and Kersters 1991a, 446VP. In G. M. Garrity, D. J. Brenner, N. R. Krieg, & J. T. Staley (Eds.), Bergey's Manual of Systematic Bacteriology (vol 2, 2ed, pp. 732–735). New York: Springer.

Williams, M. M., Domingo, J. W. S., Meckes, M. C., Kelty, C. A., & Rochon, H. S. (2004). Phylogenetic diversity of drinking water bacteria in a distribution system simulator. *Journal of Applied Microbiology*, *96*, 954–964. https://doi.org/10.1111/j.1365-2672.2004.02229.x

Wozniak, R. F., & Waldor, M. K. (2010). Integrative and conjugative elements: mosaic mobile genetic elements enabling dynamic lateral gene flow. *Nature Reviews. Microbiology*, *8*(AuGuST), 552–563. https://doi.org/10.1038/nrmicro2382

Wu, H., Wang, A. H., & Jennings, M. P. (2008). Discovery of virulence factors of pathogenic bacteria. *Current Opinion in Chemical Biology*, *12*(1), 1–9. https://doi.org/10.1016/j.cbpa.2008.01.023

Xi, C., Zhang, Y., Marrs, C. F., Ye, W., Simon, C., Foxman, B., & Nriagu, J. (2009). Prevalence of antibiotic resistance in drinking water treatment and distribution systems. *Applied and Environmental Microbiology*, *75*(17), 5714–5718. https://doi.org/10.1128/AEM.00382-09

Yabuuchi, E., Kosako, Y., Yano, I., Hotta, H., & Nishiuchi, Y. (1995). Transfer of Two *Burkholderia* and An *Alcaligenes* Species to *Ralstonia* Gen. Nov.: Proposal of *Ralstonia pickettii* (Ralston , Palleroni and Doudoroff 1973) Comb . Nov . and *Ralstonia solanacearum* (Smith 1896) Com. Nov. and *Ralstonia eutropha* (Davis

1969) C. *Microbiol. Immunol*, *39*(11), 897–904.

Yamamoto, M., Nagao, M., Hotta, G., Matsumura, Y., Matsushima, A., Ito, Y., … Ichiyama, S. (2012). Molecular characterization of IMP-type metallo- b - lactamases among multidrug-resistant *Achromobacter xylosoxidans*. *Journal of Antimicrobial Chemotherapy*, (May), 2110–2113. https://doi.org/10.1093/jac/dks179

Yang, C., & Li, Y. (2011). *Chromobacterium violaceum* infection : A clinical review of an important but neglected infection. *Journal of the Chinese Medical Association*, *74*(10), 435–441. https://doi.org/10.1016/j.jcma.2011.08.013

Yang, H. C., & Rosen, B. P. (2016). New mechanisms of bacterial arsenic resistance. *Biomedical Journal*, *39*(1), 5–13. https://doi.org/10.1016/j.bj.2015.08.003

Yang, Y., Liu, W., Xu, C., Wei, B., & Wang, J. (2017). Chemosphere Antibiotic resistance genes in lakes from middle and lower reaches of the Yangtze River , China : Effect of land use and sediment characteristics. *Chemosphere*, *178*, 19–25. https://doi.org/10.1016/j.chemosphere.2017.03.041

Yao, J., & Allen, C. (2007). The plant pathogen *Ralstonia solanacearum* needs aerotaxis for normal biofilm formation and interactions with its tomato host. *Journal of Bacteriology*, *189*(17), 6415–6424. https://doi.org/10.1128/JB.00398-07

Yoon, S.-H., Ha, S.-M., Kwon, S., Lim, J., Kim, Y., Seo, H., & Chun, J. (2017). Introducing EzBioCloud: A taxonomically united database of 16S rRNA and whole genome assemblies. *International Journal of Systematic and Evolutionary Microbiology*. https://doi.org/10.1099/ijsem.0.001755

Zankari, E., Hasman, H., Cosentino, S., Vestergaard, M., Rasmussen, S., Lund, O., … Larsen, M. V. (2012). Identification of acquired antimicrobial resistance genes. *J Antimicrob Chemother*, *67*(11), 2640–2644. https://doi.org/10.1093/jac/dks261

Zellweger, C., Bodmer, T., Täuber, M., & Mühlemann, K. (2004). Failure of ceftriaxone in an intravenous drug user with invasive infection due to *Ralstonia pickettii*. *Infection*, *32*(4), 246–248. https://doi.org/10.1007/s15010-004-3033-0

Zhang, L., Hinz, A. J., Nadeau, J., & Mah, T. (2011). *Pseudomonas aeruginosa* tssC1 Links Type VI Secretion and Biofilm-Specific Antibiotic Resistance. *Journal of*

Bacteriology, *193*(19), 5510–5513. https://doi.org/10.1128/JB.00268-11

Zhang, X.-X., Zhang, T., & Fang, H. H. P. (2009). Antibiotic resistance genes in water environment. *Applied Microbiology and Biotechnology*, *82*, 397–414. https://doi.org/10.1007/s00253-008-1829-z

Zhang, X., Xu, Y., He, X., Huang, L., Ling, J., Zheng, L., & Du, Q. (2016). Occurrence of antibiotic resistance genes in landfill leachate treatment plant and its effluent-receiving soil and surface water. *Environmental Pollution*, *218*, 1255–1261. https://doi.org/10.1016/j.envpol.2016.08.081

Zhou, Y., Xu, Y. Bin, Xu, J. X., Zhang, X. H., Xu, S. H., & Du, Q. P. (2015). Combined toxic effects of heavy metals and antibiotics on a *Pseudomonas fluorescens* strain ZY2 isolated from swine wastewater. *International Journal of Molecular Sciences*, *16*(2), 2839–2850. https://doi.org/10.3390/ijms16022839