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MINI-REVIEW

Methodological tools to study species of the genus *Burkholderia*

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Abstract
Bacteria belonging to the *Burkholderia* genus are extremely versatile and diverse. They can be environmental isolates, opportunistic pathogens in cystic fibrosis, immunocompromised or chronic granulomatous disease patients, or cause disease in healthy people (e.g., *Burkholderia pseudomallei*) or animals (as in the case of *Burkholderia mallei*). Since the genus was separated from the *Pseudomonas* one in the 1990s, the methodological tools to study and characterize these bacteria are evolving fast. Here we reviewed the techniques used in the last few years to update the taxonomy of the genus, to study gene functions and regulations, to deepen the knowledge on the drug resistance which characterizes these bacteria, and to elucidate their mechanisms to establish infections. The availability of these tools significantly impacts the quality of research on *Burkholderia* and the choice of the most appropriated is fundamental for a precise characterization of the species of interest.

Key points
- *Updated techniques to study the genus Burkholderia were reviewed.*
- *Taxonomy, genomics, assays, and animal models were described.*
- *A comprehensive overview on recent advances in Burkholderia studies was made.*

Keywords *Burkholderia* · Taxonomy · Genomics · Quorum sensing · Animal models

Introduction

The genus *Burkholderia* (phylum *Proteobacteria*, class β-*Proteobacteria*, order *Burkholderiales*, family *Burkholderiaceae*) was differentiated by the *Pseudomonas* one in 1992 based both on phenotypic characteristics as well as on 16S rRNA sequences, cellular lipid and fatty acid composition (Yabuuchi et al. 1992). Today, it comprises more than 120 species which live in different environments and are also able to colonize plants and animals, both in a beneficial and a pathogenic manner. Indeed, species able to promote plant growth and isolated from natural environments have been recently grouped in the genus *Paraburkholderia* (Vio et al. 2020). Another positive aspect of these bacteria is their ability to degrade a wide variety of pollutants, thus serving as bioremediation agents. On the other hand, the genus encompasses plant and animal pathogens, which can be host-specific or not. As an example, *Burkholderia cepacia*, which was firstly described as responsible for onion soft rot (Burkholder 1950), is also an opportunistic pathogen for cystic fibrosis and chronic granulomatous disease patients since it can cause a fatal pneumonia known as "the cepacia syndrome." It belongs to the *Burkholderia cepacia* complex (Bcc) that, together with *Burkholderia mallei* (the etiological agent of glanders) and *Burkholderia pseudomallei* (which causes melioidosis) represent the most clinically relevant species.

This mini-review is a comprehensive summary of the most recent advances in the setup of methods useful to study not only the classification but also the main features of the *Burkholderia* genus, including the genome and the drug resistance.

Since the taxonomy of *Burkholderia* has changed very much in the last years, this mini-review will start with a

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paragraph describing the old and more recent techniques used for the classification of these bacteria.

Another peculiarity of *Burkholderia* bacteria is their large genome, typically composed of two or three chromosomes, containing more than 5,000 genes (www.burkholderia.com). An important tool to understand their function is gene deletion: here we review some methods developed to allow gene deletion and silencing.

Unfortunately, *Burkholderia* pathogenic species share another common feature: drug resistance. Besides the "classical" mutations in genes encoding drug targets and the production of antibiotic modifying enzymes, the poor membrane permeability, the presence of efflux pumps, as well as the ability to form biofilm have been reported as drug resistance mediators in these bacteria. Since this renders the treatment of infections particularly challenging, here we will describe different methodologies which can contribute to find therapies alternative to the existing ones. These include the use of transposon libraries as a tool for drug discovery, the implementation of different types of assays for the characterization of quorum sensing, a process playing a central role in gene regulation and representing a key target for the development of antivirulence compounds, and the employment of animal models useful for the elucidation of the establishment of infections.

Traditional and new techniques to describe the taxonomy of the *Burkholderia* genus

The accurate identification of bacterial species belonging to the *Burkholderia* genus is very important, as they are considered opportunistic pathogens that cause life-threatening infections to immunocompromised patients. Moreover, the increasing resistance of these bacteria to many antibiotics implies the need for fast and reliable identification methods (Israyilova et al. 2016; Trespidi et al. 2020). The greatest obstacle in the correct identification of these pathogens is the high similarity among these species (Furlan et al. 2019; Jin et al. 2020; Martinucci et al. 2016; Wang et al. 2020). Different methods can be used for the identification of these bacteria (Table 1), such as the sequencing of *16S rRNA*, *23S rRNA*, *recA*, *hisA*, *rpsU* genes, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Wong et al. 2020; Fergusson et al. 2020; Gassiep et al. 2019; Furlan et al. 2019; Haeckl et al. 2019; Frickmann et al., 2014).

The sequencing of the *16S rRNA* and *23S rRNA* genes are extensively applied for bacterial identification in most laboratories. Despite studies have indicated that these methods can efficiently identify *Burkholderia* genus among other genera, with this method it is not possible to distinguish within the species (Furlan et al. 2019; Jin et al. 2020; Peeters et al. 2016). Jin et al. (2020) explored that *16S rRNA* gene has limited taxonomic resolution in the identification of *Burkholderia cepacia* complex (Bcc) species, in line with the results from other reports (Furlan et al. 2019). By the modification of Selective Medium-design Algorithm Restricted by Two constraints (SMART) protocol, a selective isolation strategy for *Burkholderia* strains was developed. This new method is based on *16S rRNA* and multiplex PCR sequencing for the genus level identification, with reports of 95% accuracy for bacterial samples (Haeckl et al., 2019).

The *recA* gene was considered another well-known molecular marker promising for accurate identification of *Burkholderia* species, but differentiation from *non-Burkholderia* species is tricky with this approach (Furlan et al. 2019; Wong et al. 2020; Depoorter et al. 2020).

hisA gene encodes a protein involved in histidine biosynthesis, which is used to distinguish species within the

Table 1 Methods used to describe the taxonomy of the *Burkholderia* genus

Method	Accuracy	References
16S rRNA and *23S rRNA* gene sequencing	No distinction within species	Furlan et al. 2019; Jin et al. 2020; Peeters et al. 2016
Selective medium-design algorithm restricted by two constraints	95%	Haeckl et al., 2019
recA gene sequencing	93.08–100%	Furlan et al. 2019; Wong et al. 2020; Depoorter et al. 2020
hisA gene sequencing	91.76–100%	Jin et al. 2020
rpsU gene sequencing	No distinction within species	Frickmann et al., 2014
Multilocus sequence typing/analysis (MLST/MLSA)	No distinction within species	Jin et al. 2020; Furlan et al. 2019
Raman spectroscopy (RS)	90–100%	Stöckel et al., 2015; Kloß et al. 2015; Moawad et al. 2019
Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)	97–100%	Fergusson et al. 2020; Wong et al. 2020; Depoorter et al. 2020; Gassiep et al. 2019; Wattal et al. 2017
Liquid chromatography-mass spectrometry multiple reaction monitoring (LC–MS/MS MRM)	97–100%	Wang et al. 2020

Burkholderia genus. It was reported that the accuracy of *recA* sequences ranged from 93.08 to 100% for 116 Bcc isolates, while *hisA* sequences identity of 115 strains ranged between 91.76 and 100% (Jin et al. 2020). However, phylogenetic trees relying on *recA* and *hisA* genes revealed better results than *16S rRNA*, although some extent of confusion and discordance was detected in the *recA* and *hisA* phylogenies (Jin et al. 2020).

rpsU sequencing method allows identification of the *Burkholderia* genus, but another molecular procedure is required for the identification at the species level of identification (Frickmann et al., 2014). Multilocus sequence typing/analysis (MLST/MLSA) is based on the nucleotide sequences of the alleles in order to show similarities within strains belonging to different species and genera. Studies revealed that sequence typing (ST) is not well characterized yet because of the great genetic variation among *Burkholderia* species, so it is suggested to use other molecular techniques able to detect diversity among these species (Jin et al. 2020; Furlan et al. 2019).

Raman Spectroscopy (RS) is a modern technique which differentiates bacteria by combining the spectral information of samples with machine learning validated reference spectra. The technique relies on the unique molecular composition showed by bacteria owing different phenotypes, which leads to minimal but important differences in their corresponding Raman spectra. Indeed, RS determines vibrational modes of molecules which provide a structural fingerprint to identify the molecules themselves. Since RS efficiency is low, long measurement times are needed to reach high identification accuracies (Ho et al. 2019). RS is different from other applied tools—including low cost ones—performing high speed analyses and extensive range of information about the chemical composition and interaction between the biomolecules within the microbes are available (Stöckel et al. 2015; Kloß et al. 2015). Moawad et al. (2019) has used RS assay to identify *B. mallei*, *B. pseudomallei*, and other *Burkholderia* spp. strains: here samples were inactivated by heat instead of formaldehyde compared with the work from Stöckel et al. (2015). The accuracy of the tool is characterized by high sensitivities (≥ 95%) for the identification of *B. mallei*, *B. pseudomallei* species and is in line with the report by Stöckel et al. (2015). The *Burkholderia* strains were properly identified with sensitivities that ranged between 90 and 100% (Moawad et al. 2019).

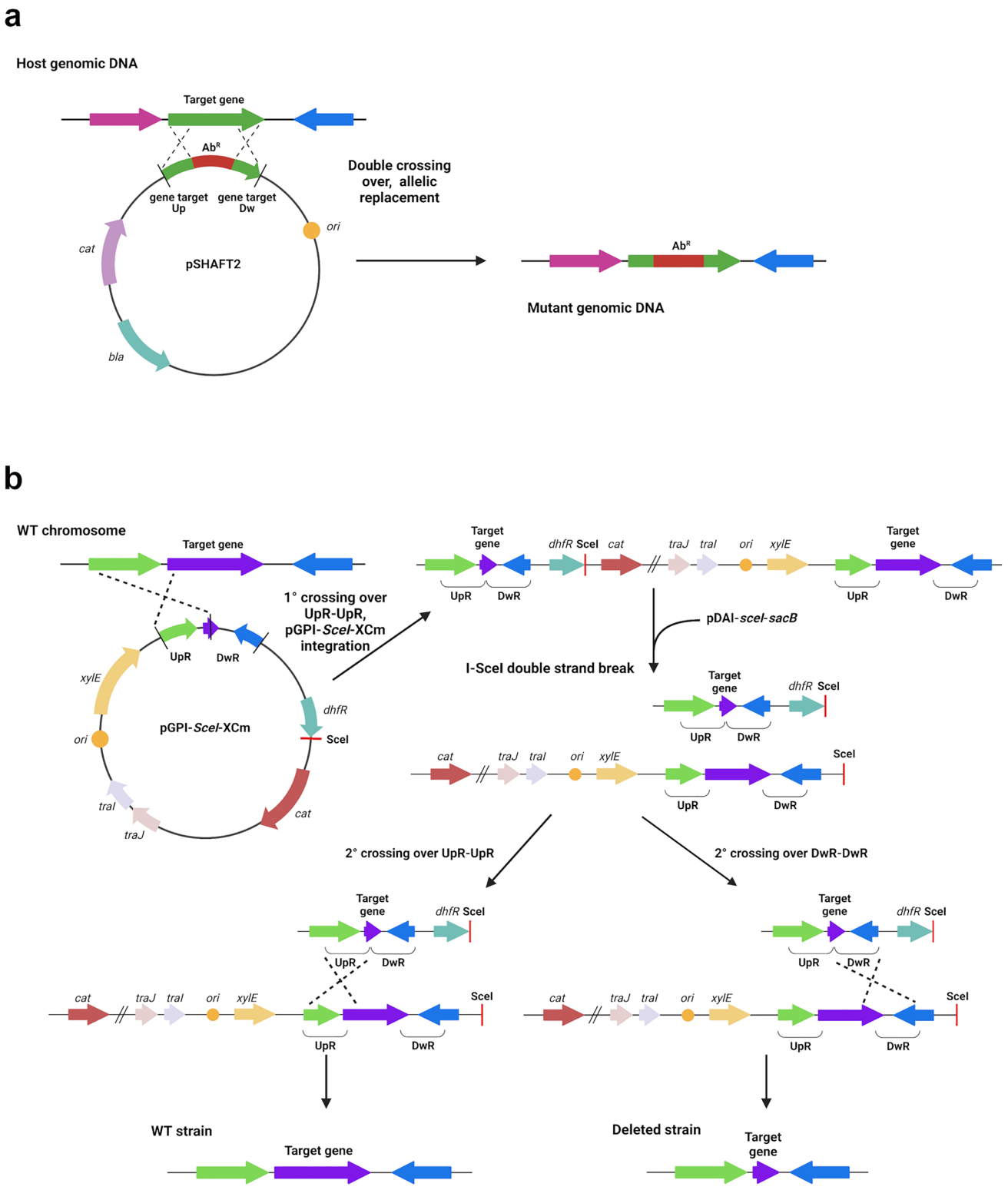
Recent automated system MALDI-TOF MS (VITEK 2, VITEK MS, and Bruker Biotyper) emerged as sensitive, practical, rapid and inexpensive tools for bacterial identification in most clinical microbiology laboratories. The identification technique is based on spectral analysis of ribosomal and heat shock proteins of the bacterial cell (Fergusson et al. 2020; Wong et al. 2020; Depoorter et al. 2020; Gassiep et al. 2019; Wattal et al. 2017). It was reported that determination of *Burkholderia* bacteria by MALDI-TOF MS at the genus level was reliable and the accuracy ranged between 97 and 100% for all bacteria samples. At the species level, the correct identification rate ranged from 26 to 83% according to the MS system, whether or not the achieved spectrum of the isolate is matched to the reference spectra included in the database (De Dios et al. 2016; Wong et al. 2020; Gautam et al. 2017; Kenna et al. 2017). Studies have shown that no automated systems could accurately identify all *Burkholderia* samples at the species level with the current database versions (Wong et al. 2020). Taking into account these results, Wang et al. (2020) developed a gene-protein based approach using Liquid Chromatography-Mass Spectrometry Multiple Reaction Monitoring (LC–MS/MS MRM) to identify Bcc: 16 different peptides were used to construct an automated multiplex LC–MS/MS MRM assay. A drawback of this assay is that the operator plays the main role in the manual curation of the phylogenetic partitions (Wang et al. 2020). Fergusson et al. (2020) have recently created the main spectral reference library for the MALDI mass spectrometer (Bruker Biotyper) for *Burkholderia* strains and related genera. As no false result was detected at the genus level, the MALDI Biotyper tool can be used even when specific species are not present in the reference library (Fergusson et al. 2020). The large scale library for *Burkholderiales* is free for use. This library and other relevant data can be downloaded (https://doi.org/10.5061/dryad.pk0p2ngjx), making the property of MALDI mass spectrometer contingent on the size and quality of the reference library.

In summary, the taxonomy of the *Burkholderia* genus changed a lot especially in the last few years, when new techniques were developed to precisely discriminate among the various species. This led to divide the genus into *Burkholderia* sensu stricto and six new other genera: *Paraburkholderia* (Sawana et al. 2014), *Caballeronia* (Dobritsa and Samadpour, 2016), *Robbsia* (Lopes-Santos et al. 2017), *Pararobbsia* (Lin et al. 2020), *Mycetohabitans*, and *Trinickia* (Estrada-de Los Santos et al. 2018). As regarding the Bcc, an update has been recently reported by Jin et al. (2020).

Understanding gene function: deletion techniques

Genetic tools are essential to study molecular mechanisms involved in cellular processes. *Burkholderia* species are characterized by high levels of antibiotic resistance, high GC content and large genomes, all features that make the editing methods designed for Gram-negative bacteria inefficient for use in *Burkholderia*.

The commonly used methods for gene deletion in *Burkholderia* are based on disruption of the targeted genes with an antibiotic-resistance marker, through an



integrative vector or by allelic replacement of the target gene with an antibiotic resistance cassette. Nowadays, the most used method for gene inactivation is based on two recombination events near the target gene, so that no vector sequences (including antibiotic-resistance cassettes) remain on the chromosome of the mutant, thus allowing the creations of multiple deletions at a time. More recently other gene silencing mechanisms for *Burkholderia* were developed, based on recombineering or on CRISPR technology.

◂**Fig. 1** Schematic diagram depicting the two gene deletion methods in *Burkholderia*: **a** gene replacement procedure described by Shastri et al. (2017). A fragment of DNA (≥ 1.0 kb) containing the target gene (or gene fragment) is cloned into a pSHAFT vector and then disrupted by insertion of an antibiotic resistance cassette, ensuring there is at least 0.5 kb of homology between the cloned DNA target region and the chromosome on either side of the cassette. The pSHAFT-derived construct is transferred into bacteria, double crossover recombinants are selected based on their resistance to the antibiotic specified by the antibiotic resistance cassette, and either sensitivity to chloramphenicol; **b** the gene replacement procedure described by Flannagan et al. (2008). The suicide vector pGPI-SceI-XCm contains regions of homology flanking the target gene in purple (UpR, green and DwR, blue). The vector is transferred into bacteria by conjugation and integrated into the chromosome by the first homologous recombination event, resulting in trimethoprim and chloramphenicol resistant merodiploids. The pDAI-SceI-SacB is transferred into the merodiploid by conjugation. The I-SceI endonuclease expressed from the plasmid introduces a double-stranded DNA break at the I-SceI recognition site on the chromosome (in red). The DNA break stimulates the second homologous recombination event through the host DNA repair system. Depending on the location of the second recombination event, the resolution of the merodiploid state either restores the wild-type allele or generates the desired gene deletion. (Created with BioRender.com)

Gene deletion based on allelic replacement allows to replace the wild type gene with a selectable resistance marker, but the use of these techniques could be problematic if the host bacteria exhibit high intrinsic resistance to antibiotics. To improve this method, Shastri et al. (2017) constructed pSHAFT-derived suicide vectors for insertional inactivation, containing the chloramphenicol resistance (Cm^R) (Fig. 1a), which allow the insertion of an antibiotic resistance cassette in the gene of interest through a double crossing over. These vectors were also improved after the substitution of the Cm^R with the GFP that allows fluorogenic detection of recombinants in strains already harboring a Cm^R (Fig. 1a) (Shastri et al. 2017). These inactivation methods were validated in *B. cenocepacia* and *B. lata* (Shastri et al. 2017).

On the other hand, the most elegant method to obtain an in-frame unmarked, marker-free, nonpolar recombination is the allelic replacement developed by Flannagan and co-workers (Flannagan et al. 2008; Hamad et al. 2010) (Fig. 1b). The regions flanking the target gene are cloned in a suicide vector called pGPI-SceI-XCm containing a SceI endonuclease recognition site (Fig. 1b). The vector is then mobilized into *Burkholderia* by triparental mating, and cointegrants are selected using trimethoprim and chloramphenicol. The plasmid pDAI-SceI-SacB, which carries the I-SceI endonuclease encoding gene, is mobilized into *B. cenocepacia* cointegrants. I-SceI produces a genomic strand break and another homologous recombination event allows bacteria to repair the break with a 50% chance of resulting in a gene deletion (Fig. 1b) (Flannagan et al. 2008; Hamad et al. 2010). The resolution of merodiploids is obtained by the excision of the integrated plasmid and the counterselection is based on tetracycline resistance and trimethoprim and chloramphenicol sensitivity.

This method was recently used to study the role of two tyrosine kinases (BCAM1331 and BceF) and of the low molecular weight protein tyrosine phosphatases BCAM0208, BceD, and BCAL2200 in pathogenesis and physiology (Andrade et al. 2016). This technique is extremely useful also in gene regulation studies, such as the characterization of the small regulatory RNA ncS35 (Kiekens et al. 2018) and of the two DNA methyltransferases (BCAL3494 and BCAM0992) involved in biofilm formation, cell aggregation, and motility (Vandenbussche et al. 2020).

This mutagenesis strategy was modified to be used with many different species of the Bcc, such as the members of the *B. cenocepacia* IIIB Midwest clones (Abdu et al. 2018). The tetracycline resistance cassette of the pDAI-SceI-SacB was substituted with a trimethoprim marker and the suicide vector was modified with only the chloramphenicol resistance cassette (Abdu et al. 2018). The protocol was used to obtain a *B. cenocepacia* PC184 strain deleted in the lipo-oligosaccharide inner core biosynthesis (Abdu et al. 2018).

The same mechanism of deletion, based on homologous recombination, was used in *B. thailandensis* to demonstrate that the ATPase of the type VI secretion system (T6SS), ClpV, has a T6SS-independent localization in the cell (Lennings et al. 2019). The ΔT6SS-5 *B. thailandensis* strain was obtained using the suicide vector pJRC115 (Lennings et al. 2019).

Moreover, this protocol was applied also in other *Burkholderia* species, such as *B. pseudomallei* and *Burkholderia glumae*. In *B. pseudomallei*, the in-frame deletion was obtained using the suicide vector pDM4 and mutants were obtained after growth on salt free LB containing 10% glucose (Wagley et al. 2017). The deletion of the glidobactin synthesis gene (*glbC*) of *B. pseudomallei* showed its involvement in bacteria survival in murine macrophages (Wagley et al. 2017). To develop a live attenuated vaccine against *B. pseudomallei*, multiple *B. pseudomallei* MSHR668 deletion mutants were constructed using the *sacB*-based vector pMo130, and exconjugants were selected on LB agar without NaCl and with 10% glucose with incubation at 25 °C for four days (Amemiya et al. 2019). In *B. glumae*, the study of the core oligosaccharide and O-antigen region was performed obtaining the deleted strains for *waaC* and *wbiFGHI* genes, using a suicide vector (Lee et al. 2019).

Another way to study gene expression and regulation in *Burkholderia* is based on the construction of conditional mutants, such as in the case of colistin resistance in *B. thailandensis*. A conditional mutant of *arn* operon was obtained with the insertion vector pSC200 that contains an inducible rhamnose promoter to regulate the expression of the operons (Panta and Doerrler 2021).

Genetic manipulation (deletion, insertion, replacement, point mutation, multi-fragment assembly and direct cloning of large fragments) can be also obtained with recombineering systems. This is based on the phage-encoded homologous recombination systems Redα/Redβ/Redγ of the lamba phage Red operon or on the RecE/RecT from Rac prophage (Li et al., 2021). Recently, this method was applied to *B. glumae* and *B. plantarii*, using three Rac bacteriophage RecET-like operons of *Burkholderia*. These recombineering systems were introduced by inserting functional promoters to activate cryptic non-ribosomal peptides. This system has the advantage of utilizing shorter homology arms (~ 50 bp) which can be included in synthetic oligonucleotides (Li et al., 2021).

Finally, Hogan et al. (2019) developed a CRISPR interference technology for gene silencing in *B. cenocepacia*, *B. multivorans* and *B. thailandensis*. The modulable expression was obtained by placing a *dcas9* from *Streptococcus pyogenes* under the control of a rhamnose inducible promoter (Hogan et al. 2019). This system was tested on the phenylacetic acid degradation operon (*paaABCDE*) and on *phbC* and *fliF* genes, which were all successfully silenced (Hogan et al. 2019).

Another example of genetic manipulation in *Burkholderia* species is the deletion of the entire chromosome three in *B. cenocepacia*. The Bcc genome consists of three replicons that were considered chromosomes because they apparently carried essential genes and each of them carried at least one rRNA operon (Agnoli et al., 2012). However, during a transposon library mutagenesis study, Agnoli and coworker isolated *B. cenocepacia* H111 mutants attenuated in virulence that had lost chromosome three. Later, using a chromosome three mini-replicon to cure chromosome three from strains of Bcc species by plasmid incompatibility, they obtained nine Bcc strains without the chromosome three (Agnoli et al., 2012). This experiment demonstrated that the chromosome three carries virulence, secondary metabolites and other accessory functions in Bcc bacteria and it is not an essential chromosomal element, rather a large plasmid (Agnoli et al., 2012).

Transposon libraries as a tool for drug discovery

The combination of large-scale transposon mutagenesis with next-generation sequencing (known as Transposon-insertion sequencing (TIS) approach) allows performing global, high-throughput analyses of bacterial genomes to define the essentiality and/or fitness contribution of each genetic feature. TIS methods are based on the preparation of saturated transposon mutant libraries in which each cell carries a single insertion. In the final population, each genetic feature is disrupted multiple times at different sites. Direct sequencing of the transposon–genome junctions, followed by reads mapping on the genome, allows determining the sites of transposon integration. As inactivation of essential genes or functions will result in the loss of the corresponding mutants, features for which mutants cannot be recovered are assumed to be essential (Cain et al. 2020). Importantly, the identification of genes encoding functions that are essential for cell growth and viability is not only important to understand the basic biological processes required to support life, but it is a key strategy to identify novel antimicrobial drug targets (Cardona et al. 2018; Hogan et al. 2018).

By using variations of the TIS experimental approach, 505 essential genes were identified in *B. pseudomallei* (Moule et al. 2014), 406 in *B. thailandensis* (Baugh et al. 2013) and 508, 383 and 339 in *B. cenocepacia* K56-2, J2315 and H111 strains, respectively (Baugh et al. 2013; Moule et al. 2014; Wong et al. 2016; Gislason et al. 2017; Higgins et al. 2017). Interestingly, despite a good overlap in the core essential genome, strain-specific differences could be observed among the analyzed closely related *B. cenocepacia* strains. Despite some of these dissimilarities can be ascribed to methodological modifications among studies, other discrepancies reflect strain-specific characteristics (Gislason et al. 2017; Higgins et al. 2017). The comparison of the available essential genomes of *B. cenocepacia*, *B. pseudomallei*, and *B. thailandensis* allowed the identification of a subset of 141 conserved essential genes (Gislason et al. 2017; Higgins et al. 2017). This conserved core essential genome of *Burkholderia* is enriched in genes encoding functions involved in the maintenance of the cell envelope, including the biosynthesis of lipid A, peptidoglycan and 4-amino-4-deoxy-arabinose (Ara4N) (Gislason et al. 2017). Importantly, while peptidoglycan and lipid A biosynthesis are essential in many Gram-negative bacteria, the essentiality of Ara4N is a peculiarity of *Burkholderia* species. Moreover, three of the common essential genes, encoding for a lysophospholipid transporter (LplT), a porin (OpcP) and a protein involved lipid A modification by L-Ara4N, were discovered as uniquely essential in the four *Burkholderia* strains analyzed, supporting their possible use as *Burkholderia*-specific targets for antibiotic treatment (Gislason et al. 2017).

An important implication of TIS methods is that the generated libraries of transposon mutants can be subjected to growth under various selective conditions to discover genes involved in niche adaptation, pathogenicity and virulence. Genomic regions showing a decreased frequency in transposon insertions over the experimental selection are assumed to be important for growth in the tested conditions. On the contrary, an increase in insertion frequency is associated to genomic features with a disadvantageous effect in the test conditions (Cain et al. 2020).

By using this approach, the identification of the insertion sites of mutants negatively selected during in vivo infection

allowed to successfully identify virulence factors of *B. pseudomallei* (Moule et al. 2016). Also, genes important for rhizosphere colonisation and phenol degradation were identified in *Burkholderia vietnamiensis* (O'Sullivan et al. 2007). The comparison of the insertion profiles of transposon mutants of *B. cenocepacia* J2315 grown in undefined rich nutrient medium (LB) and in M9 minimal medium led to the identification of additional 439 genes found to be crucial for the growth of this bacterium under conditions of nutrient depletion. These included several genes involved in aromatic amino acid biosynthesis (Wong et al. 2016). Genes essential for growth of *B. cenocepacia* H111 in minimal medium and under microoxic conditions (0.5% O_2) were similarly identified (Higgins et al. 2017).

An alternative strategy to identify conditional essential genes in *B. cenocepacia* was developed and used to detect targets of antibiotics. A library of *B. cenocepacia* K56-2 conditional growth mutants was created by large-scale delivery of a transposon element carrying an outward tightly regulated *Escherichia coli* rhamnose-inducible promoter. As transcription of target genes is allowed only in the presence of rhamnose, mutants carrying insertion in essential genes will not grow in the absence of the inducer (Cardona et al. 2006; Bloodworth et al. 2013). Moreover, mutants underexpressing essential genes encoding for targets of molecules with antibacterial activity will show increased sensitivity to the corresponding inhibitor compounds (Bloodworth et al. 2013). Libraries of conditional growth mutants therefore represent an excellent tool to identify targets of antibiotics with unknown mechanism of action. This competitive fitness screen method allowed the identification of the *B. cenocepacia* cell division protein FtsZ as the target of C109, a benzothiadiazole derivative with a broad-spectrum antibacterial activity (Hogan et al. 2018). Similarly, a *B. cenocepacia* two-component system whose inactivation confers hypersensitivity to different classes of antibiotics was found and identified as an optimal target to contrast antibiotic resistance (Cardona et al. 2018).

Despite being limited by the inability to detect non-protein targets of antibiotics, competitive growth of conditional growth mutants under selective conditions allows a rapid screening of many antimicrobial compounds, thus addressing the bottleneck of target identification (Farha and Brown 2016).

Assays to study quorum sensing

Burkholderia species exploit the cell density-dependent quorum sensing (QS) signaling to synchronize the bacterial population gene expression in response to microenvironment stimuli. As nearly all known Gram-negative bacteria, they mainly possess LuxI/LuxR-like QS systems relying on acyl-homoserine lactone (AHL) autoinducers (Slinger et al. 2019). However, other kind of QS signals are also used by *Burkholderia* spp. for cell-to-cell communication, including the *Burkholderia* diffusible signal factor (BDSF) of *B. cenocepacia* (Suppiger et al. 2016) and the 2-alkyl-4(1*H*)-quinolones (AHQs) of *B. pseudomallei* and *B. thailandensis* (Coulon et al. 2019). Altogether these form an incredibly complex signalling network that controls hundreds of genes, in particular those encoding virulence factors. Given the importance of the QS regulation in *Burkholderia* spp., and the emerging interest in this pathway as an alternative antivirulence target, several methods have been used over the years to study it (Table 2).

A widely used experimental strategy to detect the presence of AHLs in bacterial cultures involves the use of *Agrobacterium tumefaciens* reporter strains (defective for the production of the autoinducer) carrying plasmids that contain a *traG*::*lacZ* fusion, as well as the *traR* gene. Indeed, *traG* transcription is strictly regulated by the activator TraR, which is activated by the interaction with the autoinducer (an AHL molecule). Since TraR responds to a variety of AHLs, the presence of AHL molecules can be detected through β-galactosidase assays or using solid media supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). This technique was exploited by many groups, among them Cui and collaborators who studied the effect of a BDSF analogue on the AHL production of *B. cenocepacia* H111 by β-galactosidase assay (Cui et al. 2019). Two other groups used *A. tumefaciens* to visualize AHL spots from culture extracts directly on thin layer chromatography (TLC), by overlaying agar medium containing the reporter and incubating overnight, to quantify the signal production in *B. cenocepacia* and *B. lata* mutants of QS regulatory genes (Veselova et al. 2016; Jung et al. 2017).

A. tumefaciens bioassay is often used concurrently with the *Chromobacterium violaceum* CV026 assay since the two methods are complementary. Indeed, by coupling them it is possible to detect a wider variety of AHLs, given that CV026 and *A. tumefaciens* strains respond to AHLs with N-acyl side chains from C_4 to C_8 and C_6 to C_{12} in length, respectively. *C. violaceum* naturally produces the purple pigment violacein, but the CV026 strain (a violacein-negative mutant) restores the pigment production only in the presence of exogenous AHL signals, becoming suitable for an easy QS molecules detection on solid media. Concerning this, the abovementioned studies used this coupled approach on agar plates (Veselova et al. 2016) or on TLC (Jung et al. 2017) highlighting the different AHLs production in their strains. Nonetheless, *C. violaceum* can be also used alone as an indicator strain in agar well-diffusion assays, for instance to test the quorum quenching activity of *B. cepacia* (Malešević et al., 2020). This technique was used to study the poorly characterized QS systems of the plant pathogens

Table 2 Methods used to study QS systems in *Burkholderia* spp

Methods	*Burkholderia* species	Aim	References
A. tumefaciens reporter strains	*B. cenocepacia*; *B. lata*	AHLs detection	Veselova et al. 2016; Jung et al. 2017; Cui et al. 2019
C. violaceum CV026 assay	*B. cenocepacia*; *B. lata*; *B. cepacia*; *B. glumae*; *B. gladioli*	AHLs detection	Veselova et al. 2016; Jung et al. 2017; Elshafie et al. 2019; Seynos-García et al., 2019; Kang et al. 2019; Gnanasekaran et al. 2020; Malešević et al., 2020
X. campestris FE58 biosensor	*B. cenocepacia*	DSFs detection	Cui et al. 2018
lacZ transcriptional fusions	*B. cenocepacia*; *B. pseudomallei*	QS-regulated genes transcription studies	Yang et al. 2017; Klaus et al. 2018; Jenul et al. 2018; Cui et al. 2018; Cui et al. 2019; Slinger et al. 2019; Wang et al. 2021
Luciferase and GFP transcriptional fusions	Bcc species; *B. pseudomallei*; *B. thailandensis*	DFSs detection and QS-regulated genes transcription studies	Suppiger et al. 2016; Le Guillouzer et al. 2017; 2018; Waldron et al. 2019; Oppy et al. 2019; Srisanga et al. 2019; Le Guillouzer et al. 2020
LC–MS/MS	*B. cenocepacia*; *B. ambifaria*; *B. thailandensis*	AHLs and BDSF quantification	Chapalain et al. 2017; Le Guillouzer et al. 2017; 2018; Lightly et al. 2019; Waldron et al. 2019; Cui et al. 2019; Le Guillouzer et al. 2020
DCPIP enzymatic assay and nondenaturing gel electrophoresis	*B. cenocepacia*	Determination of CepI and DfsA ($RpfF_{Bc}$) catalytic activity	Spadaro et al. 2016; Scoffone et al. 2016; Buroni et al. 2018; Sass et al. 2019; Lightly et al. 2019; Waldron et al. 2019

B. glumae and *Burkholderia gladioli*. Indeed, recent studies exploited the bioassay to measure the AHLs production of a laboratory-evolved clone (Gnanasekaran et al. 2020) and an activated methyl cycle mutant of *B. glumae* (Kang et al. 2019) on TLC. Regarding *B. gladioli*, the *C. violaceum* assay was carried out on agar medium, to analyze the AHL synthesis in mutants of putative QS genes (Seynos-García et al. 2019), but also to find uncharacterized QS genes by using a cosmid genome library conjugated in CV026 reporter strain and selecting the transconjugants showing restored violacein production (Elshafie et al. 2019).

Since many *Burkholderia* species produce DSF analogues, as the BDSF (Suppiger et al. 2016), the DSF biosensor *Xanthomonas campestris* FE58 is considered a useful tool for QS research. FE58 is a strain deficient for the DSF production, which carries an *engXCA* promoter (DSF-inducible)–*gusA* (*E. coli* β-glucuronidase) fusion on the pLAFR3 vector. Thus, when FE58 is plated on a medium containing 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-glu) it is possible to visualize the presence of exogenous DSF signals by measuring the blue halo surrounding the colonies of the producer. This method was exploited by Cui and collaborators to find novel regulators involved in the *B. cenocepacia* H111 QS pathways by screening a Tn5 mutant library (Cui et al. 2018).

Beside the above described reporter microorganisms, the *lacZ* transcriptional fusion technique is widely used in QS research. Indeed, its simple use and versatility make it one of the most employed molecular methods, which allows an easy visualization of the results by β-galactosidase or X-gal assays. This approach was widely used in *B. cenocepacia* H111 where the plasmid pME2-*lacZ*, containing different QS-regulated promoters, was conjugated directly in the investigated strain to clarify the role of a two component system on QS regulation (Cui et al. 2018) or to determine the effect of a new BDSF analogue (Cui et al. 2019). Also, the same protocol was used by Jenul and collaborators to demonstrate that valdiazen is a novel QS signal in *B. cenocepacia* (Jenul et al. 2018). A *lacZ* reporter was exploited also to screen a mutant library of *B. cenocepacia* in order to find new regulators of QS systems by selecting mutants defective for QS regulatory genes (Yang et al. 2017; Wang et al. 2021). Since QS circuit is the result of an extremely complicated network of interacting proteins, sometimes having redundant roles, the use of *lacZ* fusions in *E. coli* reporters can be useful to detect specific biological activities in a heterologous background to prevent the interference of additional uncharacterized regulators in the results. For this reason, Klaus et al. used an *E. coli* reporter to elucidate the role of *B. pseudomallei* AHLs molecules in the activation of the transcription factor MalR (Klaus et al. 2018). Instead, Slinger et al. exploited this strategy to screen a library of AHL analogs and find modulators of the *B. cenocepacia* CepR in an *E. coli* reporter defective for its LuxR-type regulator (Slinger et al. 2019).

Recently, a useful biosensor for the detection of DSF signals was developed in *B. cenocepacia* H111 using the sensor plasmid pAN-L15 (Suppiger et al. 2016). This broad host range vector has the luminescence genes *luxAB* under the control of the *bclACB* promoter (strongly activated by BDSF), allowing its transcription only in the presence of a DSF signal. To avoid self-activation of the reporter genes, the plasmid was introduced in a *B. cenocepacia* H111 strain deficient for the $rpfF_{bc}$ gene. The biosensor can be used in liquid culture and in cross-streak assays and it is able to respond to several fatty acids with *cis-2* configuration even at nM concentrations (Suppiger et al. 2016; Waldron et al. 2019). Since BDSF is involved in biofilm formation, the same research group created a transcriptional fusion of the *bclACB* promoter with an unstable variant of the GFP to visualize the BDSF production within biofilms, although this biosensor resulted less sensitive than the *luxAB*-dependent one (Suppiger et al. 2016). Other examples of luminescence-dependent reporters relying on the promoterless *luxCDABE* operon are present in literature, such as the plasmid pMS402 used in *B. cenocepacia* to easily detect changes in *cepI* and *cepR* transcription (Oppy et al. 2019), the pSB401 in *E. coli* to quantify the AHL production in *B. pseudomallei* (Srisanga et al. 2019), and the mini-CTX-lux integration vector in *B. thailandensis* to shed light on its complex QS circuitry (Le Guillouzer et al. 2017; 2018; 2020).

Biological reporters are reliable tools in QS studies but, for a more precise identification and quantification of autoinducers, the use of more powerful analytical techniques is often necessary. In this case, liquid chromatography with tandem mass spectrometry (LC–MS/MS) is currently the method of choice, allowing the fine separation of the culture supernatant extracts on a HPLC column, followed by the sensitive detection of signal molecules such as AHLs (Lightly et al. 2019; Chapalain et al. 2017; Le Guillouzer et al. 2017; 2018; 2020) or BDSF (Waldron et al. 2019; Cui et al. 2019) by mass spectrometer.

The characterization of QS mechanisms goes also through the study of the enzymes involved, and in particular of the autoinducer synthases. To this end, different enzymatic assays were used to determine the catalytic activity of these proteins, as well as to test the potentiality of new inhibitors. To test the catalytic activity of *B. cenocepacia* CepI, a spectrophotometric assay that measures the formation of the *holo*-acyl carrier protein (ACP) during the biosynthetic reaction was used. Indeed, through a redox reaction between the released free thiol of ACP and the dichlorophenylindophenol (DCPIP) it is possible to determine the catalytic activity by measuring the absorbance at 600 nm (Scoffone et al. 2016; Buroni et al. 2018; Sass et al. 2019; Lightly et al. 2019). Similarly, the thioesterase activity of the other main

B. cenocepacia QS synthase DsfA ($RpfF_{Bc}$), responsible for the BDSF biosynthesis, was assessed using the DCPIP reagent (Spadaro et al. 2016), or alternatively by checking the *holo*-ACP release with conformation-sensitive nondenaturing gel electrophoresis (Spadaro et al. 2016; Waldron et al. 2019).

Animal models to study *Burkholderia* infections

The use of different biological models to study armful *Burkholderia* genus bacteria has been fundamental to acquire nowadays' knowledge on virulence and pathogenesis, mostly in the field of cystic fibrosis; different organisms are available today, differing in their complexity, research adaptability, and required laboratory setting.

Invertebrate infection models lacking the adaptive immune system can be used to study both cellular and humoral innate immune response, which involves antimicrobial peptides production, phagocytosis, and hemolymph clotting (Wong et al. 2018). *Caenorhabditis elegans* is one of the first tested invertebrate hosts to support *Burkholderia* infection (Köthe et al. 2003); the availability of tools for its genetic manipulation makes it an attractive organism for the identification of virulence factors and their conservation among different species. Moreover, its transparency can be employed to monitor bacterial localization and invasion, using GFP tagged cells (Köthe et al. 2003). Two methods can be applied to study *Burkholderia cepacia* complex infection (Tedesco et al. 2015): the "slow-killing" one produces a persistent intestinal infection as the nematode is fed with bacteria present in the nematode growth medium (NGM). Results can be observed from 1 to 3 days after larvae inoculation when bacteria eventually colonized the gut causing worms death and recalling an infection-like process (Wong et al. 2018; Tedesco et al. 2015). The second one, called "fast-killing," is used to identify potential toxins as bacteria are grown on a filter posed on a high-osmolarity medium (e.g., PGS) (Wong et al. 2018; Tedesco et al. 2015); the filter is then removed, and the larvae are added to the plate. Within 24 h of incubation, the produced toxins can temporarily paralyze *C. elegans* and sometimes kill it (Wong et al. 2018; Tedesco et al. 2015). In-liquid analysis involves *C. elegans* interaction with the pathogen of interest in the presence or absence of antibiotics or quorum sensing inhibitors (Scoffone et al. 2016), and can be extended to study *B. mallei* and *B. pseudomallei* as well (Trottmann et al. 2019; Chen et al. 2019); again, virulence is quantitatively measured. Unfortunately, past studies highlighted *C. elegans* poor correlation with both mammalian and other alternative models (Uehlinger et al. 2009; Seed and Dennis 2008), producing contradictory and mixed results; this variation is probably linked to the two killing methods displayed by the bacteria and to factors such as the age of the worms and the medium used.

Drosophila melanogaster, having a fully sequenced genome, has emerged as an alternative and easy to manipulate model (Allen et al. 2016; Castonguay-Vanier et al. 2010): its innate immune system is very similar to the human one, allowing the identification of common immunity factors via gene microarrays. To infect fruit flies, the nicking method proved to be effective, highly reproducible, and accurate (Castonguay-Vanier et al. 2010), as a needle dipped into a bacterial suspension is used to prick *Drosophila* in the thorax. Furthermore, to study Bcc infection outcomes in *D. melanogaster* on different dietary regimens, Allen et al. (2016) directly injected the bacterial suspension inside the flies using a sterile capillary needle. According to the strain, from 50 h to 6 days may be needed to record flies' death (Allen et al. 2016; Castonguay-Vanier et al. 2010).

One of the probably most used insect hosts for *Burkholderia* genus infection is *Galleria melonella*. This model provides a fast, simple, and cost-effective platform, as larvae do not need to be fed and an output is available in 72 h (Costabile et al. 2020; Srinon et al. 2019; Fathy et al., 2019; Papp-Wallace et al. 2017; Seed and Dennis 2008). Bacteria inoculation requires an injection in one of the hindmost prolegs, while pathogenesis signs can be easily followed, as they involve melanization and motility impairment; finally, infection in *Galleria* is sensitive as in mammalian models, producing comparable results with the rat agar beads model for lung pathology (Seed and Dennis 2008).

Higher organism models of infection include the *Danio rerio* embryo model. With an early development innate immune system, it is useful to study bacteria survival after phagocytosis; in addition, its immune response shows significant homology to the major inflammatory factors involved in humans, as the Toll-Like Receptors (TLRs) and the complement system (Gomes et al. 2018). Unfortunately, this model cannot fully resemble a cystic fibrosis-like infection: Vergunst et al. (2010) proved that upon infection many phagocytes recruited and activated were macrophages, showing a bias towards this response. As for nematodes, zebrafish embryos are transparent, providing a valuable tool to follow invasion and dissemination over time, while bacteria are inoculated through the caudal vein and results may be available within 24 h post-infection (Gomes et al. 2018).

To conclude, the most complete biological model to study *B. cepacia* complex infection is the murine one, with a total of 15 mice engineered to further study the different disease degrees of cystic fibrosis (O'Neal et al. 1993; Ratcliff et al. 1993; Dorin et al. 1994; Colledge et al. 1995; Hasty et al. 1995; van Doorninck et al. 1995; Zeiher et al. 1995; Delaney et al. 1996; Rozmahel et al. 1996; Fisher et al. 2012). These mice models can be divided into two categories: the first

one is originated with a "replacement strategy," with mice unable to produce CFTR at all (Snouwaert et al. 1992; Dorin et al. 1994); the second one, originated with an "insertional strategy," replicates the known clinical mutations with disease severity, survival rates, and pathology unique to each model (Ratcliff et al. 1993; O'Neal et al. 1993; Colledge et al. 1995; Hasty et al. 1995; van Doorninck et al. 1995; Zeiher et al. 1995; Delaney et al. 1996; Rozmahel et al. 1996). On the other hand, many of these mice developed a range of intestinal complications that would cause fatality: to ameliorate this problem, Durie et al. (2004) produced a long-lived C57BL/6 J *Cftr*$^{-/-}$ mouse displaying human lung pathology and progressive multi-organ affliction. Despite the efforts, none of the previous models could mimic a spontaneous bacterial infection: factors involved may be the relatively short life span of *Cftr*$^{-/-}$ mutants, living in a sterile environment, the smaller airway size, and the presence of a non-CFTR calcium-activated Cl^- channel, all conditions which are not comparable to human (Wilke et al. 2011; Clarke et al. 1994). Bcc bacteria are also important pathogens in patients affected by the autoimmune chronic granulomatous disease (CGD), which impairs phagocytosis and bacterial clearance: to this end, gp91$^{phox-/-}$ and p47$^{phox-/-}$ mice were generated (Pizzolla et al. 2012; Sousa et al. 2007). Compared to a CF background, they present bacteria persistence in both macrophages and neutrophils but greatly differ in the mucociliary clearance and in the compartmentalized infection. Finally, to establish a chronic, non-lethal lung *B. cepacia* infection, the rat agar beads model in C57BL/6 J mice can be employed (Bragonzi et al. 2017): the use of bacteria imbedded in agar beads is useful to study tissue invasion and histopathology in WT and *Cftr*$^{-/-}$ mice. Beads are inoculated intratracheally and mice are sacrificed after 72 h. This model resembles a highly neutrophilic infiltrate in the lung and a marked pulmonary infection and inflammation, fully overlapping the devastating condition found in cystic fibrosis patients (Bragonzi et al. 2017).

All the previously considered *Burkholderia* species (including *B. mallei* and *B. pseudomallei*) can be intratracheally or intranasally inoculated in BALB/c and C57BL/6 J mice to assess survival, proving how these models are useful also for vaccine development (Saikh et al. 2019; Amemiya et al. 2020; Khakhum et al. 2019; Sabet and Griffith, 2020; Vanhoutte et al. 2017).

Conclusions

Bacteria belonging to the *Burkholderia* genus are heterogeneous and versatile, able to survive in different environments and to cause diseases in plant and animal hosts. The study and characterization of these microorganisms is complicated due to their large genomes and drug resistance. Here we reviewed the main techniques used in the last few years to try to elucidate the taxonomy, genomics, virulence, resistance and ability to cause infections of these opportunistic pathogens. Taxonomy is evolving very fast thanks to new tools which lead to the precise identification of species: this is not easy because of the similarity among genomes, but very important to properly characterize bacteria. Indeed, the *Burkholderia* genus was initially classified as *Pseudomonas*, but many differences exist between these pathogens.

At the same time, gene deletion techniques and transposon libraries revealed essential to properly assess gene functions and find novel drug targets. This is complicated by the poor genetic manipulation of some species; nonetheless, important progresses have been made in the last few years.

The study of quorum sensing has the double advantage of leading to the understanding of gene regulation and of characterization of new potential antivirulence targets, being this pathway involved in the regulation of the expression of virulence-related genes. In this way, the choice of the best assay is fundamental.

Finally, an overview of animal models suitable to study the ability of *Burkholderia* bacteria to cause infections has been reported. Also in this case, many alternatives are available and the choice should be performed based on the strain, disease to be studied, but also costs.

Overall, design and optimization of innovative procedures are fundamental to get new insights into *Burkholderia* bacteria and this review should have given a comprehensive summary of the most useful tools.

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Declarations

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