Genomics reveals the novel species placement of industrial contaminant isolates incorrectly identified as *Burkholderia lata*

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Abstract

The *Burkholderia cepacia* complex (Bcc) is a closely related group of bacteria, composed of at least 20 different species, the accurate identification of which is essential in the context of infectious diseases. In industry, they can contaminate non-food products, including home and personal care products and cosmetics. The Bcc are problematic contaminants due to their ubiquitous presence and intrinsic antimicrobial resistance, which enables them to occasionally overcome preservation systems in non-sterile products. *Burkholderia lata* and *Burkholderia contaminans* are amongst the Bcc bacteria encountered most frequently as industrial contaminants, but their identification is not straightforward. Both species were historically established as a part of a group known collectively as taxon K, based upon analysis of the *recA* gene and multilocus sequence typing (MLST). Here, we deploy a straightforward genomics-based workflow for accurate Bcc classification using average nucleotide identity (ANI) and core-gene analysis. The workflow was used to examine a panel of 23 *Burkholderia* taxon K industrial strains, which, based on MLST, comprised 13 *B. lata*, 4 *B. contaminans* and 6 unclassified Bcc strains. Our genomic identification showed that the *B. contaminans* strains retained their classification, whilst the remaining strains were reclassified as *Burkholderia aenigmatica* sp. nov. Incorrect taxonomic identification of industrial contaminans is a problematic issue. Application and testing of our genomic workflow allowed the correct classification of 23 Bcc industrial strains, and also indicated that *B. aenigmatica* sp. nov. may have greater importance than *B. lata* as a contaminant species. Our study illustrates how the non-food manufacturing industry can harness whole-genome sequencing to better understand antimicrobial-resistant bacteria affecting their products.

**DATA SUMMARY**

The authors confirm that all supporting data, code and protocols have been provided within the article. Illumina raw sequence reads and associated genomes have been deposited in the European Nucleotide Archive (ENA) under ENA project accession number PRJEB42964, with the exception of BCC1315, which was previously submitted under accession number ERA2359236.

**INTRODUCTION**

The *Burkholderia cepacia* complex (Bcc) is a group of closely related Gram-negative bacteria with a continuously evolving taxonomy, and currently comprises 20 named species [1, 2]. They have a diverse range of importance, including causing infection in people with cystic fibrosis (CF) [3] and plant pathogenesis [4], and, in relation to biotechnology, having roles in bioremediation and biological control [5] and being problematic industrial contaminants [6, 7]. Members of the Bcc are difficult to identify using traditional
phenotypic microbiological techniques, as exemplified by their initial classification as *Pseudomonas cepacia* up until their taxonomic reclassification in 1997 [8]. *Bcc* bacteria often require selective media for their initial isolation [9], followed by molecular techniques such as 16S rRNA and *recA* gene sequencing [10], and multilocus sequence typing (MLST) [11] for accurate identification. Despite the widespread application of these molecular marker sequencing techniques, multiple novel species groups exist within the *Bcc* [1, 12].

MLST schemes have been developed for a multitude of bacteria of interest, including the *Bcc* [13]. MLST was designed to enable the unambiguous epidemiological characterization of closely related bacteria based on polymorphisms at seven conserved gene loci, from which bacteria are assigned a sequence type (ST) [14]. MLST analysis has previously identified the predominant STs amongst the *Bcc* in the context of CF infections, particularly global surveillance of the dissemination of ST types amongst CF cohorts [15, 16]. Moreover, MLST is a powerful tool for industry, where *Burkholderia* pose a contamination risk due to their intrinsic antimicrobial and biocide resistance [17]. For example, *Burkholderia* isolates caused 3% of European recalls due to microbial contamination of non-sterile non-food products between 2005 and 2018 [6]. Industrial microbiology and its associated identification standards are primarily based on low-resolution identification methods. Such techniques include culture enrichment and biochemical profiling [18], rapid but non-specific approaches such as ATP production assessment [19], or molecular techniques for general microbial identification, such as 16S rRNA gene sequencing [20]. These techniques are insufficient for accurate identification of certain *Bcc* species. The accurate characterization of bacteria of interest is a cornerstone of epidemiological investigations to ensure effective tracking, typing, and control. This issue is exemplified by the proportion of microbial contaminants reported as unidentified in international databases for non-food product recalls, at 49% of incidents [6]. However, with increased affordability and application of whole-genome sequencing [21], industry may now readily access the higher level of resolution afforded by genomics. In the case of the *Bcc* species, this has resulted in the discovery of novel species groupings, such as within *Burkholderia cenocepacia* [22], where genomics reclassified isolates identified as *B. cenocepacia* III-B by *recA* and MLST analysis [23, 24] as the proposed novel species *Burkholderia servocepacea* [22].

Taxon K (also known as group K) is a multi-species grouping within the *Bcc* that was proposed in 2009 based on analysis of the *recA* gene and MLST, and within which two novel species were formally named as *Burkholderia contaminans* and *Burkholderia lata* [25]. Subsequently, in 2013, *B. lata* was identified as one of the predominant *Bcc* species found as an industrial contaminant [26], and the model environmental *B. lata* type strain 383T was shown to be capable of adapting its tolerance to a range of in-use preservatives, such as the isothiazolinones [26]. In a 2020 taxonomic study, Depoorter et al. [27] observed that *Bcc* taxon K bacteria split into at least three main species clades, encompassing the originally named species *B. lata* and *B. contaminans*, but also revealing *Burkholderia aenigmatica* sp. nov. as a new taxon with sufficient genetic and phenotypic differences to support its proposal as a new species. Going forward, the accurate identification of this preservative-tolerant species group in the industrial environment is crucial. In relation to *Bcc* industrial contaminants, the performance of existing molecular techniques such as *recA* and MLST that had been used to identify them [26] needs to be re-evaluated, particularly in light of further novel species being proposed in the taxon K group [27].

To date, few studies of non-food product microbial contaminants have made use of genomics to determine the species identity of problematic contaminants [6]. Herein, we use genomics to provide extensive taxonomic characterization of a panel of 23 *Bcc* isolates recovered from industrial contamination incidents, and previously identified as taxon K by MLST analysis. We show that the resolution provided by genomic taxonomy techniques accurately reclassifies industrial taxon K isolates within novel or existing species groups compared to MLST. Finally, we provide a straightforward genomics workflow for bacterial taxonomic identification in the context of industrial microbiology, which expands on past criteria using 16S rRNA gene and functional gene sequence analysis [28], and incorporates average nucleotide identity (ANI) analysis for greater resolution [29]. Application of this workflow to *Bcc* bacteria and taxon K species found as industrial contaminants provided...
Table 1. Genome-sequenced industrial Burkholderia cepacia complex taxon K isolates used for identification workflow. Genome-derived sequence types (STs) highlighted in bold are known to comprise *B. aenigmatica* sp. nov, based on the findings of Depoorter et al. [27]. The type strain *B. aenigmatica* LMG 13014^T^ (accession: SAMEA5795692) was used for ANI comparison to the species-level identity. Historical IDs were obtained from the Cardiff Bcc collection database. ‘ANI m vs MLST type strain’ denotes the ANIm value obtained when comparing to the type strain of the species proposed by historical MLST ID.

| Strain    | Genome size | Coverage | Assembly accession | Historical ID     | Current MLST-assigned taxon* | Historical ST | Genome-derived ST | ANI (%) vs MLST type strain | ANI (%) vs *B. aenigmatica* | ANI ID          |
|-----------|-------------|----------|--------------------|-------------------|-----------------------------|---------------|-------------------|-----------------------------|-----------------------------|--------------------------|
| BCC1282   | 7.32        | 9.1      | SAMEA7997990       | Bcc novel taxon K | *B. aenigmatica*            | 333           | 333               | NA                          | 99.9                       | *B. aenigmatica*          |
| BCC1284   | 8.37        | 11.0     | SAMEA7997991       | *B. lata*         | *B. aenigmatica*            | 98            | 98                | 94.6                       | 95.7                       | *B. aenigmatica*          |
| BCC1285   | 8.49        | 12.2     | SAMEA7997992       | *B. lata*         | *B. aenigmatica*            | 98            | 98                | 94.7                       | 95.8                       | *B. aenigmatica*          |
| BCC1288   | 6.31        | 6.1      | SAMEA7997993       | *B. lata*         | *B. aenigmatica*            | 98            | 98                | 94.8                       | 95.7                       | *B. aenigmatica*          |
| BCC1289   | 7.34        | 29.9     | SAMEA7997994       | *B. lata*         | *B. aenigmatica*            | 98            | 98                | 94.7                       | 95.7                       | *B. aenigmatica*          |
| BCC1290   | 8.26        | 12.2     | SAMEA7997995       | *B. lata*         | *B. aenigmatica*            | 98            | 98                | 94.8                       | 95.8                       | *B. aenigmatica*          |
| BCC1294   | 8.77        | 12.0     | SAMEA7997996       | *B. lata*         | *B. aenigmatica*            | 98            | 98                | 94.7                       | 95.8                       | *B. aenigmatica*          |
| BCC1296   | 7.55        | 10.4     | SAMEA7997997       | *B. lata*         | *B. aenigmatica*            | 119           | 119               | 94.7                       | 95.8                       | *B. aenigmatica*          |
| BCC1297   | 7.94        | 8.4      | SAMEA7997998       | *B. lata*         | *B. aenigmatica*            | 119           | 119               | 94.8                       | 95.8                       | *B. aenigmatica*          |
| BCC1298   | 6.47        | 12.2     | SAMEA7997999       | *B. lata*         | *B. aenigmatica*            | 119           | 119               | 94.8                       | 95.8                       | *B. aenigmatica*          |
| BCC1299   | 5.69        | 19.9     | SAMEA7998000       | *B. lata*         | *B. aenigmatica*            | 119           | 119               | 94.8                       | 95.7                       | *B. aenigmatica*          |
| BCC1300   | 5.74        | 6.8      | SAMEA7998001       | Bcc novel taxon K | *B. aenigmatica*            | 334           | 334               | NA                         | 97.6                       | *B. aenigmatica*          |
| BCC1302   | 6.23        | 13.0     | SAMEA7998002       | Bcc novel taxon K | *B. aenigmatica*            | 333           | 333               | NA                         | 99.9                       | *B. aenigmatica*          |
| BCC1303   | 6.12        | 12.6     | SAMEA7998003       | Bcc novel taxon K | *B. aenigmatica*            | 333           | 333               | NA                         | 99.9                       | *B. aenigmatica*          |
| BCC1313†  | 6.51        | 12.1     | SAMEA7998004       | Bcc novel taxon K | NA                         | 335           | 335               | NA                         | 98.8                       | *B. aenigmatica*          |
| BCC1314‡  | 5.13        | 17.5     | SAMEA7998005       | Bcc novel taxon K | NA                         | 336           | 336               | NA                         | 97.5                       | *B. aenigmatica*          |
| BCC1315   | 8.01        | 37.4     | SAMEA6503210       | *B. contaminans*   | *B. contaminans*            | 943           | 943               | NA                         | 94.1                       | *B. contaminans*          |
| BCC1321   | 4.43        | 17.2     | SAMEA7998006       | *B. lata*         | *B. aenigmatica*            | 339           | 339               | 94.9                       | 95.8                       | *B. aenigmatica*          |
| BCC1323‡  | 4.71        | 8.5      | SAMEA7998007       | *B. contaminans*   | NA                         | 323           | 323               | 97.4                       | 94.4                       | *B. contaminans*          |
| BCC1406   | 4.29        | 5.8      | SAMEA7998008       | *B. lata*         | *B. aenigmatica*            | 103           | 103               | 94.7                       | 96.0                       | *B. aenigmatica*          |
| BCC1554   | 7.31        | 25.3     | SAMEA7998009       | *B. lata*         | *B. aenigmatica*            | 119           | 119               | 94.7                       | 95.8                       | *B. aenigmatica*          |
| BCC1582‡  | 6.36        | 16.5     | SAMEA7998010       | *B. contaminans*   | NA                         | 102           | 102               | 99.9                       | 94.5                       | *B. contaminans*          |
| BCC1595‡  | 5.86        | 20.0     | SAMEA7998011       | *B. contaminans*   | NA                         | 482           | 482               | 98.6                       | 94.3                       | *B. contaminans*          |

*As reported by Depoorter et al. [27].
†ST does not have a species assigned within pubMLST.
‡Did not have a sequence type assigned prior to this study.
an accurate and simple means of identification of these problematic micro-organisms.

**METHODS**

**DNA extraction and genome sequencing**

Isolates were revived via plating onto tryptic soya agar, followed by inoculation of 3 ml tryptic soya broth for overnight culture, and were pelleted before genomic DNA was extracted. DNA extraction was performed on a panel of 23 historical contaminant isolates stored in the BCC collection at Cardiff University, using a Maxwell 16 Tissue DNA Purification kit and instrument (Promega, UK). These isolates were identified as a part of taxon K (Table 1) by MLST [26]. Within this panel, of 13 isolates that had previously been identified as *B. lata*, 4 were classified as *B. contaminans* and 6 were identified as a novel species subgroup within taxon K [26]. After Nextera XT (Illumina, UK) DNA library preparation, paired-end sequencing (150 bp) was performed on extracted genomic DNA using an Illumina NextSeq 500 platform at the Cardiff School of Biosciences Genomics Research Hub.

**Genome assembly, and development of a taxon K database for genomic analyses**

Illumina adaptors were trimmed from 150-nucleotide paired-end reads using the TrimGalore script, and genomes were assembled using SPAdes v3.14.0 [30] and/or Unicycler v0.4.8 [31]. Assembled contigs were then screened for contamination using Kraken2 v2.0.8 [32]. A taxon K database was developed using 58 genomes, including the genomes sequenced in this study, and all available genomes in the ENA database for taxon K and related species, obtained using enaBrowserTools v1.5.4 (available via GitHub, https://github.com/enaBrowserTools). This database included the complete genomes for type strains of *B. contaminans* (LMG 6992<T>, *B. lata* (383<T>, and the newly sequenced type strain of *B. aenigmatica* (LMG 13014<T>) [27].

**Determining the species-level identity of taxon K isolates**

ANI analysis was conducted upon the taxon K genomes using the Python script PyANI v0.2.7 (available via GitHub, https://github.com/widdowquinn/pyani), using both BLASTN (generating an ANIb value) and MUMmer (generating an ANIm value) for alignment [31]. As defined previously, ANIb refers to pairwise ANI values calculated using the blastn algorithm, whilst ANIm refers to pairwise ANI results calculated with the MUMmer algorithm. Heatmaps were also generated using PyANI to provide a visualization of the level of identity between taxon K genomes. An ANI threshold of 95% was used for analyses [29, 33], and genomes with an ANI value >95% in comparison to a taxonomic type strain were designated as matching that species.

MLST profiles were generated using MLSTCheck [34] (available via GitHub: https://github.com/sanger-pathogens/mlst_check), which uses BLASTN to compare a query genome to PubMLST databases. All 58 genomes in the taxon K database were annotated using Prokka (v1.14.5) [35] under default parameters, before Roary (v3.12.0) [36] was used to generate a core-gene alignment of 255 genes, using the outputs of Prokka. The concatenated allele alignment produced by MLSTCheck
was then used to generate a concatenated MLST allele phylogeny, whilst the 255 core-gene alignment produced by Roary was used to generate a phylogeny using RAxML [37]. A GAMMA model of rate heterogeneity supported by 100 bootstraps was used for both phylogenetic trees. Phylogenetic trees were then visualized using FigTree v1.4.2 [38] and edited using Inkscape v0.91 [39].

RESULTS

ANI analysis confirms that the industrial isolates historically identified as *B. lata* or Bcc novel taxon K belong to *B. aenigmatica* sp. nov.

Of the 23 industrial isolates subjected to genomic analysis, ANI-based species-level identification (Fig. 1) was only concordant with the genomes previously classified as *B. contaminans* by MLST (Fig. 2a). All four industrial isolates displayed ANI values above the 95.0% threshold [33] for species identification (ANI=97.4–99.9%) when compared to the *B. contaminans* LMG 23361 T species reference (Fig. 1). In contrast, all 13 industrial isolates identified as *B. lata* by MLST displayed ANI values below the threshold for species delineation (ANI=94.6–94.9%, see Table S2, available in the online version of this article) in comparison to the *B. lata* 383 T species reference. Interestingly, the most closely related complete genome by ANI was that of the *B. lata* A05, a strain of industrial origin, isolated from a mouthwash containing 0.2% of the preservative chlorhexidine [40]. Comparing strain A05 to *B. lata* 383 T gave an ANIm of 94.6% (Table S2), but strain A05 displayed ANIm values of 98.6–99.7% (Table S2) in comparison to the 13 industrial isolates, suggesting that it had been incorrectly designated as *B. lata*. ANI also revealed the existence of several other incorrectly classified/species borderline taxon K genomes, originating from non-industrial sources and labelled as *B. contaminans* or *B. lata* within the database (see Fig. 1, Table S1).

Recently, the novel Bcc species *B. aenigmatica* has been proposed after phenotypic and genomic examination of taxon K isolates [27]. Comparison to the *B. aenigmatica* type strain LMG 13014 T revealed that the six unidentified taxon K industrial isolates belonged to *B. aenigmatica* (ANI=97.5–99.9%, Table S2). This was also supported by the MLST sequence types matching those of known *B. aenigmatica* types (Table 1; see below). The remaining 13 isolates previously identified as *B. lata* formed a *B. aenigmatica*-like species grouping (ANI=95.7–96.0%, Table S2). Using a 95% ANI threshold, these isolates were designated as *B. aenigmatica*. It is important, however, to note that this ANI value is set arbitrarily based upon previous analyses of prokaryotic genomes [33], and that ambiguity exists around this threshold. As such, true genomic delineation should be achieved using values derived from the context of a given dataset (e.g. 97% for taxon K, see the Discussion).

Comparison of MLST and core-gene phylogenomics clarifies past misidentification of taxon K isolates and identifies *B. aenigmatica* as a problematic contaminant

MLST analysis based on *in silico* gene extraction from the genomes of all industrial isolates corroborated their historical ST identification (Table 1). Phylogenetic analysis of the concatenated MLST alleles placed the four *B. contaminans* industrial isolates [26] alongside the *B. contaminans* type strain LMG 23361 T (Fig. 2a). This MLST analysis also placed the 13 industrial isolates previously identified as *B. lata* [26], the *B. aenigmatica* [27] type strain, and the additional *B. lata*
Fig. 3. Proposed workflow for the identification of *Burkholderia cepacia* complex species. The analysis workflow is dependent upon an initial identification by multilocus sequence typing in order to provide a preliminary species-level identification, before moving to identification using genome to genome comparison by means of average nucleotide identity and core-genome phylogenetic comparisons to confirm species nomenclature.
industrial isolate A05 [40], as adjacent to the B. lata species type strain 383\(^3\) (Fig. 2a).

To improve the phylogenomic resolution, core-gene analysis was used to generate an alignment of 255 core-genes for all taxon K genomes examined (Fig. 2b). The core-gene alignments provided an altered phylogenetic topological definition that contrasted with the MLST phylogeny but corroborated ANI analysis (Fig. 1a, b). The five B. contaminans isolates still placed alongside the type strain in this analysis. All 13 industrial isolates defined as B. lata by MLST (Fig. 1a) were clustered within a phylogenetically distinct group (shaded box, Fig. 2b), which did not include the type strain B. lata 383\(^3\). A number of isolates that clustered within this clade possessed STs (ST98, ST119, and ST333, Table 1) that are now recognized as belonging to B. aenigmatica sp. nov [27]. Moreover, a distinct phylogenetic split exists within this clade, with B. aenigmatica and B. aenigmatica-like isolates forming a closely related subclade, with B. lata A05 being the most phylogenetically similar complete genome. This split is supported by both ANIb (Fig. 1a) and ANIm (Fig. 1b), where B. aenigmatica and B. aenigmatica-like isolates form two distinct alignment clusters. Overall, core-gene phylogenetic analysis was concordant with both ANIm and ANIb, and the designation of 6 taxon K isolates and 13 taxon K isolates [26] as B. aenigmatica. This dataset also revealed a unique intraspecies sub-clading, for which a limited amount of evidence was observed in previous datasets [27].

**DISCUSSION**

Overall, the data presented illustrate a clear workflow for the identification of Bcc species as important industrial contaminants and clinical bacteria. This genomic workflow expands on past minimum taxonomic criteria [26] and presents a straightforward series of analyses that can accurately classify taxonomically complex bacteria such as Bcc species (Fig. 3). It supports the view that although MLST, as one of the most widely applied methods in relation to identification of Bcc bacteria, can be used to generate a preliminary profile of isolates of interest and group them into the Bcc, further genomic analysis is required to accurately classify isolates within certain groups such as taxon K. MLST was not able to accurately identify the taxon K industrial isolates correctly to the species level (Fig. 2a). This is evidenced by the core-gene phylogenetic analysis and ANI, placing 19 taxon K isolates previously identified as B. lata or as unidentified by MLST into B. aenigmatica sp. nov. This distinction is not shown by BLAST or phylogenetic analysis of MLST genes, both of which place these isolates alongside the B. lata type strain, 383\(^3\), or do not provide species-level identification at all.

Although a 95–96% ANI threshold [33] is accepted as a primary delineator of bacterial species identity, and it could be argued that the 13 B. aenigmatica-like isolates belong the B. aenigmatica sp. nov., ANI thresholds should be chosen as appropriate for a given dataset, as it is also known that there is genetic discontinuity around this boundary [29]. In the context of this taxon K dataset, we chose 95% as the threshold for ANI, thereby classifying all 19 isolates as B. aenigmatica. However, examining higher or lower ANI boundaries for different groups of bacteria may aid classification (Fig. 1). In the case of Bcc taxon K strains, we observed that a 97% ANI threshold provided additional support to the separation of B. lata, B. contaminans and novel species isolates within the taxon K contaminant collection. This is clearly shown in our analyses, which demonstrate a strong concordance between core-gene phylogenetic clading (see Fig. 2b), and ANI (Fig. 1a, b), where ANI value ranges are shown for each species grouping) if a 97% threshold was to be used.

To conclude, techniques that analyse differences throughout the genome, as opposed to between a limited number of conserved genes such as MLST, are essential to accurately identify Bcc species going forward. To this end, core-gene analysis [41] and ANI [29, 33] should be considered the gold standard techniques for the accurate identification of taxon K species. Furthermore, given that all of the B. lata industrial isolates sequenced in this study and the mislabelled deposited industrial strain A05 genome (Tables 1 and S1) are members of the new taxa B. aenigmatica [27], the identity of all industrial and clinical strains reported as B. lata should be re-evaluated. This is an important identification correction in relation to industrial microbiology, as from 2013 B. lata was observed to be the most common species present in a collection of 60 contaminant strains [26]. The fitness of B. aenigmatica sp. nov. in relation to preservative tolerance and survival within industrial products remains to be determined. However multiple members of the taxon K group are intrinsically resistant to antimicrobials and have been identified as problematic contaminants [6, 7, 26]. The elucidation of this novel taxonomic grouping B. aenigmatica playing a role in contamination, and the contribution of a large panel of industrial genomes to public databases, will be invaluable in the further development of the taxonomy of Burkholderia species.

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**Author contributions**

We describe author contributions to the paper using the CRediT taxonomy. Conceptualization: E. C.-O. and E. M. Data curation: E. C.-O. Formal analysis: E. C.-O. Funding acquisition: E. M., T. R. C., T. P. and B. M. Investigation: E. C.-O. and E. M. Methodology: E. C.-O. Project administration: E. M. Resources: E. M., T. R. C. Software: E. C.-O. Supervision: E. M., T. R. C., T. P., S. C.-L. and B. M. Validation: E. C.-O. Visualization: E. C.-O. Writing – original draft: E. C.-O. and E. M. Writing – review and editing: all authors.

**Conflicts of interest**

At the time of analysis, T. P. was an employee of Unilever R&D, and B. M. and S. C.-L. are employees of Unilever R&D, the company that part-funded the studentship to E. C.-O. E. C.-O is currently an employee of Unilever R&D. The remaining authors have no conflicts of interest.
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