MICROBIAL GENOMICS

RESEARCH ARTICLE

Price *et al.*, *Microbial Genomics* 2020;6

DOI 10.1099/mgen.0.000406

Duplex real-time PCR assay for the simultaneous detection of *Achromobacter xylosoxidans* and *Achromobacter* spp.

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Abstract

Several members of the Gram-negative environmental bacterial genus *Achromobacter* are associated with serious infections, with *Achromobacter xylosoxidans* being the most common. Despite their pathogenic potential, little is understood about these intrinsically drug-resistant bacteria and their role in disease, leading to suboptimal diagnosis and management. Here, we performed comparative genomics for 158 *Achromobacter* spp. genomes to robustly identify species boundaries, reassign several incorrectly speciated taxa and identify genetic sequences specific for the genus *Achromobacter* and for *A. xylosoxidans*. Next, we developed a Black Hole Quencher probe-based duplex real-time PCR assay, Ac-Ax, for the rapid and simultaneous detection of *Achromobacter* spp. and *A. xylosoxidans* from both purified colonies and polymicrobial clinical specimens. Ac-Ax was tested on 119 isolates identified as *Achromobacter* spp. using phenotypic or genotypic methods. In comparison to these routine diagnostic methods, the duplex assay showed superior identification of *Achromobacter* spp. and *A. xylosoxidans*, with five *Achromobacter* isolates failing to amplify with Ac-Ax confirmed to be different genera according to 16S rRNA gene sequencing. Ac-Ax quantified both *Achromobacter* spp. and *A. xylosoxidans* down to ~110 genome equivalents and detected down to ~12 and ~1 genome equivalent(s), respectively. Extensive *in silico* analysis, and laboratory testing of 34 non-*Achromobacter* isolates and 38 adult cystic fibrosis sputa, confirmed duplex assay specificity and sensitivity. We demonstrate that the Ac-Ax duplex assay provides a robust, sensitive and cost-effective method for the simultaneous detection of all *Achromobacter* spp. and *A. xylosoxidans* and will facilitate the rapid and accurate diagnosis of this important group of pathogens.

DATA SUMMARY

The GenBank and SRA accession numbers for all assemblies and raw sequence data are listed in Table S1 (available in the online version of this article).

INTRODUCTION

The genus *Achromobacter* comprises 21 officially designated species [1]. These Gram-negative non-fermentative bacteria are found ubiquitously in environmental reservoirs, including rivers, ponds, residential water sources, soil, mud and some plants [2, 3]. *Achromobacter* spp. are also important nosocomial and community-acquired pathogens, particularly in people with cystic fibrosis (CF), cancer, immunoglobulin deficiencies, renal disease, endocarditis and diabetes, and those undergoing invasive procedures [4, 5]. Members of this genus can cause a spectrum of disease, including bacteraemia, cholecystitis, endocarditis, keratitis, lymphadenitis, meningitis, osteomyelitis, peritonitis, pneumonia and urinary tract infections [6, 7]. Although several organs can be infected by *Achromobacter* spp., the respiratory and urinary tracts are the most common sites of infection [5]. *Achromobacter* spp. have been isolated from several usually sterile hospital products, such as disinfectants, ultrasound gel, dialysis fluids,

Received 27 March 2020; Accepted 26 June 2020; Published 15 July 2020

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Keywords: comparative genomics; cystic fibrosis; respiratory infections; polymicrobial infections; real-time PCR; diagnostics.

Abbreviations: Ac, *Achromobacter* sp.; ARDRA, amplified ribosomal DNA restriction analysis; Ax, *Achromobacter xylosoxidans*; BHQ, Black Hole Quencher; LoD, limit of detection; LoQ, limit of quantitation; NTC, no-template control; WGS, whole-genome sequencing.

Data statement: All supporting data, code and protocols have been provided within the article or through supplementary data files. Two supplementary tables are available with the online version of this article.

contact lens fluid, eardrops, incubators, respirators, humidifiers and deionized water, consistent with their adaptability to survive in diverse environments [2, 4, 8]. *Achromobacter* spp. are becoming increasingly common in people with CF, being cultured in up to 30 % of CF airways [9–11]. Although historically considered to be of low pathogenic potential, there is mounting evidence that CF infections caused by *Achromobacter* spp. are associated with adverse clinical presentations and outcomes, especially in immunocompromised individuals [10–13]. Therefore, their rapid identification is essential for guiding appropriate therapeutic treatments and improving patient prognosis [14].

Naturally multidrug-resistant bacteria, including *Achromobacter* spp., are increasingly being retrieved from CF airways due to the intensified implementation of aggressive antibiotic therapies [15, 16]. *Achromobacter* spp. prevalence in CF centres globally range from 3–30 % [9]; of these, between 10 and 52% progress to a chronic infection [10, 11]. In addition to their intrinsic antibiotic resistance towards aztreonam, tetracyclines, and some penicillins and cephalosporins, *Achromobacter* spp. possess a similar denitrification system to *Pseudomonas aeruginosa*, which facilitates their survival and proliferation in hypoxic and anoxic environments such as those found in CF airways [5].

Although several *Achromobacter* species can infect CF airways [12], *Achromobacter xylosoxidans* is the most common, comprising ~42–65 % of all *Achromobacter* spp. identified in CF respiratory secretions [4, 17–19]. Until recently, the role of *Achromobacter* spp. in disease pathogenesis has been unclear; however, recent studies have shown that CF patients with an *Achromobacter* spp. infection are in fact at greater risk of experiencing a pulmonary exacerbation [12], and patients with chronic infections exhibit severe airway obstruction and more rapid lung function decline [10, 11, 13]. Further, these pathogens can cause a range of serious diseases, such as pneumonia, meningitis, osteomyelitis, urinary tract infections and ocular infection, in non-CF patients [20].

Current diagnostic methods for identifying *Achromobacter* spp. and *A. xylosoxidans*, including the commonly used matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) VITEK MS and Bruker MALDI Biotyper mass spectrometry platforms, provide a reasonably accurate method for identifying these organisms [21, 22]. However, all *Achromobacter* spp. are allocated as *A. xylosoxidans*/*A. denitrificans* on the VITEK MS platform [23], thus providing limited capacity for accurate species-level identification. The Bruker platform has the ability to discriminate six *Achromobacter* species (*A. denitrificans*, *A. insolitus*, *A. ruhlandii*, *A. piechaudii*, *A. spanius* and *A. xylosoxidans*) [21], although it suffers from some false-positive and false-negative errors [24, 25]. VITEK MS and other mass spectrometry-based platforms require a purified isolate to obtain an accurate speciation result, which limits the utility of this platform, as it cannot be used directly on polymicrobial clinical specimens such as sputum, resulting in longer turnaround times, potentially incorrect antimicrobial treatment (e.g. using aminoglycosides to treat inherently resistant *A. xylosoxidans* infections [26]), and higher costs [27, 28]. In addition, mass spectrometry-based equipment has a large upfront cost and footprint, rendering this method out-of-reach for smaller, less-resourced laboratories. To address this shortcoming, an automated multiplex PCR has recently been developed to detect four non-fermentative Gram-negative bacterial species, including *A. xylosoxidans*, directly from respiratory samples using the BD MAX System [23]. This multiplex assay detected *A. xylosoxidans* with 97 % specificity, but only 78 % sensitivity [23], indicating suboptimal diagnosis of this organism using this method. Further, the BD MAX multiplex assay was not designed to identify other *Achromobacter* spp., meaning that ~50 % of CF infections caused by *Achromobacter* spp. cannot be diagnosed with this method. Other genotyping methods, such as amplified ribosomal DNA restriction analysis (ARDRA) [29], multilocus sequence typing [30], *nrdA* gene sequencing [19] and whole-genome sequencing (WGS), provide robust identification and speciation methods for *Achromobacter* spp., but are laborious and cannot be performed in a rapid or cost-effective manner.

Here, we used a large-scale comparative genomic approach to identify genetic loci specific to all *Achromobacter* species, and to *A. xylosoxidans* only. We subsequently designed a highly specific and accurate *Achromobacter* spp. and *A. xylosoxidans* (Ac-Ax) duplex PCR assay for the simultaneous detection of these organisms. Phylogenomic analysis of 158 global *Achromobacter* genomes representing at least 15 different species, including 65 *A. xylosoxidans* genomes, was used to robustly identify species boundaries and to reassign several incorrect taxon assignments. Candidate genetic regions specific for all *Achromobacter* spp. and for *A. xylosoxidans* were then assessed for assay design suitability, followed by Ac-Ax duplex assay development and validation on 198 isolates comprising 116 *Achromobacter* spp., 48 *A. xylosoxidans* and 34 non-*Achromobacter* spp. Finally, the Ac-Ax duplex assay was tested on 38 CF sputa DNA obtained from 21 adults, 4 of

Impact Statement

Achromobacter spp. are intrinsically multidrug-resistant bacteria that are emerging as an important cause of nosocomial and community-acquired infections. This group of pathogens is often misdiagnosed or even overlooked in clinical laboratories using current methodologies, which has major implications for patient prognosis and antimicrobial stewardship efforts. This study employed a large-scale comparative genomics approach to guide real-time PCR assay design that targeted all *Achromobacter* spp. (Ac), and *Achromobacter xylosoxidans* (Ax). We show that the Ac-Ax duplex can rapidly, accurately and inexpensively identify these pathogens from both polymicrobial specimens and purified cultures. This new duplex assay will assist in the identification of these important, yet often overlooked, multidrug-resistant organisms in both clinical and environmental settings.

whom were positive for *Achromobacter* spp. according to 16S rRNA gene metataxonomic sequencing, to determine assay specificity and sensitivity in polymicrobial specimens.

METHODS

Achromobacter spp. genomes and taxonomic reassignment

Publicly available data from 158 global *Achromobacter* genomes representing at least 15 species were downloaded from the National Center for Biotechnology Information (NCBI) GenBank and Sequence Read Archive (SRA) databases (paired-end Illumina data only) in May 2019 (Table S1). Genomes from the following species were available for this study: *Achromobacter aegrifaciens* (*n*=1), *Achromobacter agilis* (*n*=1), *Achromobacter arsenitoxydans* (*n*=1), *Achromobacter denitrificans* (*n*=10), *Achromobacter dolens* (*n*=1), *Achromobacter insolitus* (*n*=8), *Achromobacter insuavis* (*n*=2), *Achromobacter marplatensis* (*n*=3), *Achromobacter mucicolens* (*n*=1), *Achromobacter piechaudii* (*n*=3), *Achromobacter pulmonis* (*n*=1), *Achromobacter ruhlandii* (*n*=7), *Achromobacter spanius* (*n*=6), *Achromobacter veterisilvae* (*n*=1), *A. xylosoxidans* (*n*=79) and *Achromobacter* sp. (*n*=33).

Genome assemblies in GenBank that lacked corresponding raw reads in the SRA database were converted into simulated Illumina reads using ART version MountRainier [31]. Prior to comparative genomic analysis, SRA data were quality-filtered by Trimmomatic v0.33 [32] using parameters described elsewhere [33].

Comparative genomics to identify *Achromobacter* spp. and *A. xylosoxidans* loci

The methods for *in silico* identification of candidate conserved loci for *Achromobacter* spp. and *A. xylosoxidans* assay design have been detailed elsewhere [34, 35]. Briefly, phylogenomic analysis was first performed to identify the *A. xylosoxidans* species boundary and to reassign incorrect species designations, followed by identification of conserved loci for the target taxa (i.e. all *Achromobacter* spp. and *A. xylosoxidans* only) among the 158 *Achromobacter* genomes (Table S1) using default parameters embedded in the SPANDx v3.2.1 comparative genomics software. The -m flag of SPANDx was employed to identify biallelic, orthologous, core-genome single-nucleotide polymorphisms (SNPs) among all genomes [36]. Both simulated and real Illumina reads were mapped against the *A. xylosoxidans* NCTC 10807 reference genome (GenBank reference NZ_LN831029.1). Phylogenomic reconstruction was carried out on the 174 240 biallelic orthologous SNPs identified among all 158 *Achromobacter* spp. strains using the heuristic maximum parsimony function of PAUP* v4.0a.165 [37]. The resultant phylogenomic tree (Fig. 1) was bootstrapped for 1000 replicates and midpoint-rooted using FigTree v1.4.0 prior to visualization. The BEDcov output generated by BEDTools [38], which is wrapped in the SPANDx pipeline, was used to identify conserved candidate loci for subsequent real-time PCR assay design.

Identification of genetic loci specific for *A. xylosoxidans* and for all *Achromobacter* spp.

Using the BEDcov output generated by default by the SPANDx pipeline, a total of ~9 kb of DNA across four discrete loci was identified as highly conserved across all *A. xylosoxidans* strains (*n*=65), but absent or highly divergent in other *Achromobacter* spp. (*n*=93) (Table 1). The sequences for these loci were examined by microbial nucleotide BLAST (http://blast.ncbi.nlm.nih.gov; performed November 2019) to identify candidate regions for real-time PCR assay design. Using this approach, the *AT699_RS16685* locus, which encodes a hypothetical protein in *A. xylosoxidans*, was selected for assay design. The process for designing the *Achromobacter* spp. assay was different due to the need to cater for more genetic diversity across all *Achromobacter* strains. The highly conserved *rpoB* gene, which encodes DNA-directed RNA polymerase β-subunit protein, was targeted for assay design.

Ac-Ax duplex real-time PCR assay design

DNA sequences from these candidate loci for all 158 strains (for the Ac assay) and for the 65 *A. xylosoxidans* strains (for the Ax assay) were extensively assessed for specificity using microbial nucleotide discontiguous MEGABLAST (http://blast.ncbi.nlm.nih.gov/). Candidate oligo performance was assessed *in silico* using NetPrimer (http://www.premierbiosoft.com/netprimer/) and Beacon Designer (http://www.premierbiosoft.com/qOligo/Oligo.jsp/) using parameters described elsewhere [39]. The following primers and Black Hole Quencher (BHQ) probes were designed for specific *Achromobacter* spp. and *A. xylosoxidans* detection, respectively (5′ to 3′): Ac_F (CACrTAGCTCACGAACTCCAAGC), Ac_R (CAGCTTCAATCCTACCTAACTTTCCT) and Ac_probe (HEX-CGTAGCCGACGGTTTGCAGG-BHQ1), which generates a 144 bp amplicon; and Ax_F (AGCGTCACGGAATGCAGC), Ax_R (AAGGGCGTTTCAACGAGAGC) and Ax_probe (FAM-AGGTCATAGGCGTAGACCAGC-BHQ1), which generates a 127 bp amplicon.

Real-time PCR parameters

PCR optimization was performed for both assays in singleplex across a range of primer (0.2, 0.25, 0.3, 0.35 and 0.4 µm) and subsequently probe (0.25, 0.3, 0.35, 0.4 µm) concentrations to determine the optimal oligomer concentrations for each assay prior to conversion to the duplex format. The optimized Ac-Ax duplex PCR consisted of 1× Sso Advanced Universal Probes Supermix (Bio-Rad Laboratories, Gladesville, NSW, Australia), 0.40 µm of the Ax_probe, Ac_F and Ac_R oligomers, 0.35 µm Ac_probe, and 0.25 µm Ax_F and Ax_R oligomers (Macrogen, Inc., Geumcheon-gu, Seoul, Republic of Korea), 1 µl DNA template and RNase/DNase-free PCR-grade water (Thermo Fisher Scientific), to a final 5 µl reaction volume. Thermocycling was performed using the CFX96 Touch Real-Time PCR Detection System

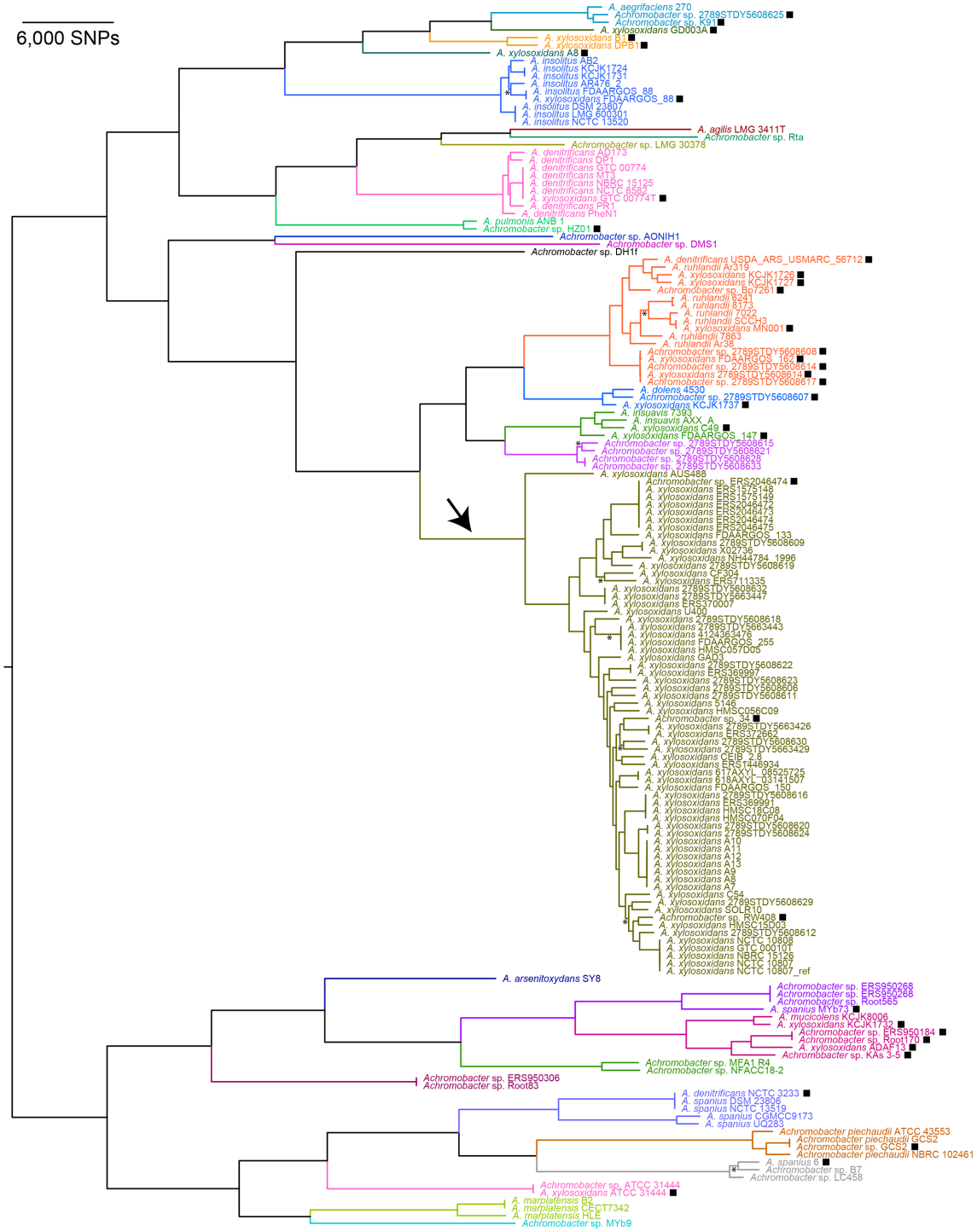

Fig. 1. Maximum parsimony phylogenomic analysis of 158 global *Achromobacter* spp. strains. Twenty-nine putative species were identified within this genus based on publicly available genomic data. The delineation separating *A. xylosoxidans* from other *Achromobacter* spp. is shown by a black arrow. Incorrectly speciated taxa are shown by a black box next to the strain name. Branches with bootstrap values with <80 % support are labelled with an asterisk. Consistency index=0.27.

(Bio-Rad), with parameters consisting of an initial hot start/denaturation step of 95 °C for 2 min, followed by 45 cycles of denaturation at 95 °C for 5 s and annealing/extension at 60 °C for 5 s. The *A. xylosoxidans* LMG 1863 type culture strain [40] was used as a control for all experiments, with no-template controls (NTCs) included in all runs to assess assay performance. All PCR results were examined using the CFX Maestro v4.1.2433.1219 software (Bio-Rad).

Table 1. Conserved loci in *Achromobacter xylosoxidans* (*n*=65 genomes) that are highly divergent or absent in other *Achromobacter* spp. (*n*=93 genomes) according to SPANDx

Genetic coordinates (NCTC 10807*)	Encoded genes	Functions
1444000.1445000	*AT699_RS06590* (partial), *AT699_RS06595* (partial)	PAS domain-containing sensor histidine kinase, sensor histidine kinase
3722000.3723000†	*AT699_RS16680* (partial), *AT699_RS16685*, *AT699_RS16690*	Glutathione S-transferase family protein, hypothetical protein, winged helix–turn–helix transcriptional regulator
4622000.4623000	*AT699_RS20725* (partial), *AT699_RS20730*	LysE family translocator, PhzF family phenazine biosynthesis protein
5775000.5782000	*AT699_RS26110* (partial), *AT699_RS26115*, *AT699_RS26120*, *AT699_RS26125*, *AT699_RS26130*, *AT699_RS26135* (partial)	Tripartite tricarboxylate transporter substrate-binding protein, hypothetical protein, D-3-phosphoglycerate dehydrogenase, porin, long-chain fatty acid CoA ligase, hypothetical protein

*GenBank reference NZ_LN831029.1.
†Locus targeted for PCR assay development in the current study.

Analysis of *Achromobacter* spp. strains using the Ac-Ax PCR assay

We examined the performance of our duplex assay across *A. dolens* LMG 26840 and *A. insuavis* LMG 26845 [41], *A. ruhlandii* LMG 1866 [42], *A. xylosoxidans* LMG 1863 [40] and 115 Australian strains identified as *Achromobacter* spp. according to: (i) VITEK MS microbiological testing (*n*=12), (ii) API 20 NE phenotypic testing (*n*=85), (iii) ARDRA (*n*=13) and (iv) WGS (*n*=5) (Table S2). Among the Australian strains, 2 were previously identified as *A. ruhlandii* (QLDACH007 and QLDACH010) and two as *A. xylosoxidans* (QLDACH001 and AUS488) according to WGS [43, 44], 1 (QLDACH016) was a novel *Achromobacter* sp. according to WGS [44] and 110 were allocated as *Achromobacter* sp. according to API 20 NE, ARDRA, or VITEK MS. Strains were grown on chocolate agar for 24 h at 37 °C prior to chelex DNA extraction, as described elsewhere [39].

Ac-Ax PCR assay sensitivity and specificity testing

To determine Ac-Ax PCR assay sensitivity, the limits of detection (LoD) and quantification (LoQ) were determined [39] in the duplex assay format using 1 : 10 serial dilutions of *A. xylosoxidans* LMG 1863 DNA ranging from 40 ng μl^{-1} to 0.04 fg/μl^{-1}, and a total of eight replicates per dilution. Twenty-four NTCs were also included. Next, the Ac-Ax duplex assay was tested for specificity against 34 non-*Achromobacter* isolates comprising *Burkholderia* spp. (*n*=3), *Enterobacter* spp. (*n*=4), *Klebsiella* spp. (*n*=3), *Prevotella* spp. (*n*=4), *P. aeruginosa* (*n*=9), *Staphylococcus* spp. (*n*=3), *Stenotrophomonas maltophilia* (*n*=1) and *Veillonella* spp. (*n*=6). These organisms were selected as they represent a cross-section of species identified in human infections, particularly in CF airways.

16s rRNA gene sequencing

DNA from 38 sputa from 21 adults with CF presenting at a single CF clinic in Brisbane, Australia, were extracted using an enzymatic lysis buffer containing 250 U ml^{-1} mutanolysin, 20 mg ml^{-1} lysozyme, 22 U ml^{-1} lysostaphin, 1.2 % Triton-X and 1× Tris-EDTA. DNA was extracted using the Qiagen DNeasy Blood and Tissue kit according to the 'Gram-positive' extraction protocol (Qiagen, Chadstone, Vic, Australia). Extracted sputum DNA was subjected to 300 bp paired-end Illumina MiSeq 16S rRNA gene metataxonomic sequencing to identify the presence of achromobacterial DNA in these polymicrobial specimens. These data were compared with those from the Ac-Ax assay to determine the performance of this duplex PCR on polymicrobial specimens. The V3–V4 region was targeted using the universal 341F (5′-CCTAYGGGRBGCASCAG) and 806R (5′-GGACTACNNGGGTATCTAAT) primers, with PCRs, sequencing and data analysis performed at the Australian Genome Research Facility (St Lucia, Qld, Australia) according to standardized workflows.

16S rRNA gene sequencing was carried out on five *Achromobacter* isolates (as determined by ARDRA or API 20 NE testing) that were negative for the Ac-Ax duplex assay to assign species designations. The ~1.3 kb 16S rDNA amplicons were generated using primers 785F (5′-GGATTAGATACCCTGGTA) and 907R (CCGTCAATTCMTTTRAGTTT), followed by dideoxy sequencing at Macrogen, Inc. (Geumcheon-gu, Seoul, Republic of Korea). Sequence chromatograms were visualized in BioEdit v7.2 [45].

16s rRNA gene universal real-time PCR

A universal bacterial 16S rRNA gene SYBR Green assay comprising oligos 16S-UniF (5′-TCCTACGGGAGGCAGCAGT) and 16S-UniR (5′-GGACTACCAGGGTATCTAATCCTGTT) [46] was used for relative bacterial DNA quantitation across the 38 CF sputa. Reactions were carried out in duplicate in a final 5 µl volume using the same master mix, real-time PCR instrumentation, and DNA volumes as described above for the Ac-Ax assay. Minor modifications were made to the thermocycling parameters as follows: initial denaturation for 2 min at 95 °C, followed by 40 cycles of denaturation for 15 s at 95 °C and 20 s annealing/extension at 60 °C. The relative abundance of achromobacterial DNA in these CF sputa was determined by subtracting the Ac assay cycles-to-threshold (C_T)

value (where positive) from the 16S rRNA gene C_T value (i.e. ΔC_T).

RESULTS

Comparative genomic analysis of *Achromobacter* spp

Phylogenomic reconstruction of the 158 *Achromobacter* genomes confirmed that all taxa were *Achromobacter* spp. However, a considerable number of taxonomic errors (*n*=36;~23%) were identified in the dataset (Fig. 1, black boxes). Taxonomic reassignment was therefore carried out to ensure correct delineation of the *A. xylosoxidans* clade from all other *Achromobacter* spp. for PCR assay design, resulting in a final dataset comprising the following: *A. aegrifaciens* (*n*=3), *A. agilis* (*n*=1), *A. arsenitoxydans* (*n*=1), *A. denitrificans* (*n*=9), *A. dolens* (*n*=3), *A. insolitus* (*n*=9), *A. insuavis* (*n*=4), *A. marplatensis* (*n*=3), *A. mucicolens* (*n*=6), *A. piechaudii* (*n*=4), *A. pulmonis* (*n*=2), *A. ruhlandii* (*n*=17), *A. spanius* (*n*=5), *A. veterisilvae* (*n*=1), *A. xylosoxidans* (*n*=65) and *Achromobacter* spp. (*n*=25) (Table S1). In total, 29 *Achromobacter* clades were identified among the 158 genomes, of which only 14 corresponded to a previously assigned species. The remaining 15 clades lack a type species genome for comparison and may therefore represent novel species (Fig. 1).

BLAST analysis identifies additional *Achromobacter* spp. and *A. xylosoxidans* misclassifications

Microbial nucleotide BLAST analysis of the Ac-Ax duplex assay amplicons against 31 complete and 141 draft *Achromobacter* spp. genomes (*n*=172), 32972 complete non-*Achromobacter* genomes, and 9040 draft betaproteobacterial genomes (as at 25 November 2019) identified a small number of strain misclassifications in the NCBI database. BLAST analysis of the 144 bp Ac amplicon identified one putative false-positive hit (*Bordetella bronchiseptica* strain KU1201; contig BBVB01000043.1); however, closer inspection showed greater homology of this contig to *Achromobacter* spp. (~95–99% identity and 97–100% coverage) than *Bordetella* spp. (~90–91% identity and ~68–71% coverage), indicating an NCBI database error for this strain. The closest non-*Achromobacter* hit for the Ac amplicon was in *Bordetella* genomosp. 7 (~89% identity and 100% coverage). Importantly, there were six SNPs in the 20 bp Ac_probe sequence in these taxa, which would inhibit their detection in the real-time PCR assay due to insufficient sequence homology. For all 172 *Achromobacter* spp. genomes, there was 100% nucleotide conservation at the primer- and probe-binding regions. Therefore, *in silico* analysis confirmed excellent specificity of the Ac assay for all known members of this genus.

For the 127 bp Ax amplicon, one putative false-positive BLAST hit was identified (*Achromobacter* sp. RW408); however, this isolate was reclassified as *A. xylosoxidans* according to our phylogenomic analysis (Fig. 1), confirming an NCBI database error for this strain. The closest non-*A. xylosoxidans* hit was in *Burkholderia mesoacidophila*, with BLAST analysis yielding 100% coverage but only 75% sequence identity in this organism. As with the Ac assay, there were six SNPs in the 21 bp Ax_probe sequence in *B. mesoacidophila*, which would inhibit detection of this non-target species due to substantial sequence diversity. All 55 *A. xylosoxidans* genomes possessed 100 % nucleotide conservation at the primer- and probe-binding sites. Therefore, this assay shows excellent *in silico* specificity for *A. xylosoxidans*.

Ac-Ax performance on *Achromobacter* isolates

Of the 119 *Achromobacter* isolates examined with the Ac-Ax duplex real-time PCR assay, 114 were Ac-positive, and among these, 48 were also Ax-positive (Table S2). There were no instances of Ax-positive but Ac-negative strains. The four type culture strains performed as expected, with all being Ac-positive, and only *A. xylosoxidans* LMG 1863 being Ax-positive; *A. insuavis* LMG 26845, *A. ruhlandii* LMG 1866 and *A. dolens* LMG 26840 failed to amplify with the Ax assay. The five isolates that did not amplify with either assay were subjected to 16S rRNA gene sequencing of a ~1.3 kb amplicon to determine their species identity. Of these, two (QLDACH029 and QLDACH035) were identified as *P. aeruginosa*, and the remaining three were identified as *B. bronchiseptica* (QLDACH105), *Cupriavidus metallidurans* (QLDACH120) and *S. maltophilia* (QLDACH125). The two *P. aeruginosa* isolates were previously identified as *Achromobacter* sp. according to ARDRA [47], whereas the other three isolates were identified as *Achromobacter* sp. according to API 20 NE. The performance of each genotyping method and concordance with the Ac-Ax duplex assay is summarized in Table 2.

Ac-Ax performance for non-*Achromobacter* isolates

Of the 34 non-*Achromobacter* species and 24 NTCs tested against the Ac-Ax duplex assay, none yielded detectable amplification (data not shown).

Ac-Ax sensitivity

The lower limits of detection (LoD) and quantification (LoQ) for the Ac-Ax duplex assay were determined on

Table 2. Summary of genotyping methods for *Achromobacter* identification and performance comparison with the Ac-Ax duplex real-time PCR assay

Initial ID method	No. Ac-positive (% concordance)	No. Ax-positive (% concordance)
WGS (*n*=5)	5 (100%)	2 (100 %)
ARDRA (*n*=13)	11 (84.6%)	NA
VITEK MS (*n*=12)	12 (100%)	NA
API 20 NE (*n*=85)	82 (96.5%)	NA

Ac, *Achromobacter* sp.; ARDRA, amplified rRNA gene restriction analysis; Ax, *Achromobacter xylosoxidans*; MS, mass spectrometry; NA, not applicable; WGS, whole-genome sequencing.

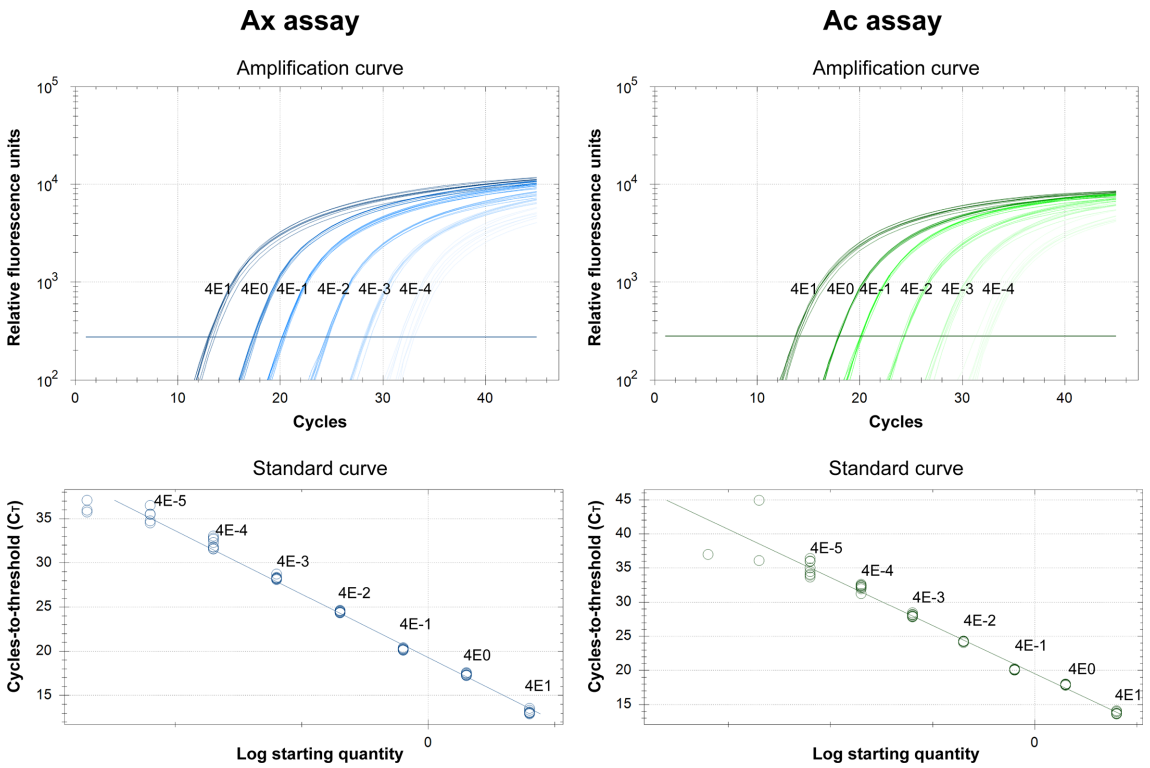

Fig. 2. Limits of detection (LoD) and quantitation (LoQ) for the *Achromobacter xylosoxidans* (Ax; left) and *Achromobacter* spp. (Ac; right) duplex real-time PCR assay across a standard curve. Genomic DNA from *A. xylosoxidans* LMG 1863 was normalized to 40 ng µl^{-1} (i.e. 4E1 ng µl^{-1}), followed by a 10-fold DNA dilution series down to 0.04 fg µl^{-1} (i.e. 4E−7 ng µl^{-1}). This DNA dilution panel was used to test LoD and LoQ limits for the Ax-Ac duplex assay.

A. xylosoxidans LMG 1863 genomic DNA obtained from a pure culture (Fig. 2). Using a 10-fold DNA dilution series ranging from 40 ng µl^{-1} to 0.04 fg µl^{-1}, the LoQ for both assays was ~400 fg µl^{-1}, or ~110 genome equivalents (GEs). The LoD values were more sensitive than the LoQ values, with an Ac assay LoD of ~40 fg µl^{-1} (~12 GEs) and an Ax LoD of ~4 fg µl^{-1} (~1 GE) (Fig. 2).

Comparison of the Ac-Ax assay and metataxonomics for *Achromobacter* identification from CF sputa

To determine its performance on polymicrobial clinical specimens, the duplex Ac-Ax PCR was tested against 38 sputa from 21 adults with CF. Of these, 5 (i.e. 15%) contained *Achromobacter* spp. at relative abundances ranging from 0.1–63.6% according to metataxonomic sequencing (Table 3), with the remaining 33 samples failing to identify any *Achromobacter* 16S rRNA gene reads. Consistent with the metataxonomic findings, 4/33 sputa were PCR-positive according to the Ac assay, and 3 of these were also Ax-positive; however, this species result could not be compared with the metataxonomic data due to insufficient species-level resolution obtained from the 16S rRNA gene V3–V4 region.

The relative abundance of achromobacterial DNA between the metataxonomic and duplex PCR methods was also consistent. For example, the highest proportion of achromobacterial DNA was detected in SCHI0009 (ΔC_T value of 2.8), which possessed the highest relative abundance of achromobacterial reads (63.6%) according to metataxonomics (Table 3). The one Ac-Ax-negative sample, SCHI0030 Day 6, only contained a relative abundance of 0.1% achromobacterial DNA in the metataxonomic sequence data. Sputa from another patient, SCHI0014, had the same low relative abundance of achromobacterial DNA but was Ac-positive; however, the C_T value (36.3) was found to be outside the LoQ and LoD values for this assay (Fig. 2), reflecting the stochastic nature of detection capability beyond these limits. The higher sensitivity of the metataxonomic method for achromobacterial detection was also expected due to the multicopy nature of the 16S rRNA gene (n=3) in *Achromobacter* spp. compared with the single-copy nature of the Ac and Ax targets.

DISCUSSION

Several phenotypic (e.g. API 20 NE, VITEK MS) and genotypic (e.g. ARDRA, gene sequencing, WGS, real-time PCR) methods are available to identify *Achromobacter* spp. These methods provide varying degrees of sensitivity, specificity, cost-effectiveness, turnaround time and resolution. The gold standard method, WGS, enables highly accurate and

Table 3. Performance comparison of the Ac-Ax real-time duplex PCR assay against 16S rDNA metataxonomic sequencing on five *Achromobacter*-positive sputa obtained from CF airways

Sample	C_T		ΔC_T*	16S rDNA metataxonomic sequencing (% relative abundance)
	Ac	Ax		
A. xylosoxidans LMG 1863	18.4	18.5	ND	NA
SCHI0014	36.3	Neg	15.2	*Achromobacter* sp. (0.1%)
SCHI0003	28.3	26.0	4.5	*Achromobacter* sp. (35.5%)
SCHI0009	25.0	25.5	2.8	*Achromobacter* sp. (63.6%)
SCHI0030 Day 1	34.7	34.7	9.3	*Achromobacter* sp. (0.4%)
SCHI0030 Day 6	Neg	Neg	NA	*Achromobacter* sp. (0.1%)

*Determined by subtracting the Ac cycles-to-threshold (C_T) from the 16S rDNA C_T.
Ac, *Achromobacter* sp.; Ax, *A. xylosoxidans*; NA, not applicable; nd, not determined; Neg, negative PCR result.

comprehensive species identification, but is currently laborious, slow (>8 h to result), costly (~AUD $80), and requires specialized bioinformatic tools and knowledge to analyse sequence data. VITEK MS has good success in identifying *Achromobacter* spp. to the genus level; however, this method currently cannot attain reliable species-level resolution, with e.g. *A. xylosoxidans* unable to be differentiated from *A. denitrificans* [23], despite these species being genetically distinct (Fig. 1).

We chose the real-time PCR platform for Ac-Ax assay development due to its multiplexing capability, low per-sample cost, high accuracy potential, direct detection from polymicrobial specimens (e.g. sputum), good sensitivity, greater accessibility in lower-resourced laboratories and rapid (same-day) turnaround time [23]. The upfront equipment cost of real-time PCR equipment (~USD $25 000–40 000) is also considerably less than that for VITEK MS (USD $200 000) or many next-generation sequencing platforms, such as Illumina, and it has a much smaller laboratory footprint. The Ac-Ax consumables cost is comparable to that for VITEK MS at ~USD $1 per sample, compared with ~USD $30 for the BD Max real-time PCR platform, making the Ac-Ax assay a cost-effective method for achromobacterial identification. The Ac-Ax assay also has the advantage of simultaneous detection of both *Achromobacter* sp. and *A. xylosoxidans*. In contrast, most existing methods only detect *A. xylosoxidans*, meaning that ~50% of *Achromobacter* CF infections remain undiagnosed with these methods [4, 17–19].

The Ac-Ax assay is highly accurate, with no false-positives or false-negatives identified according to *in silico* analysis of all microbes present in the NCBI Microbes database, or via laboratory testing of several clinically important species. Indeed, our initial *in silico* BLAST analysis of Ac and Ax targets resolved incorrect species assignments in two publicly available genomes: *B. bronchiseptica* KU1201 (actually *Achromobacter* sp.) and *Achromobacter* sp. RW408 (actually *A. xylosoxidans*), demonstrating the highly accurate nature of these targets. Laboratory testing of the Ac-Ax duplex assay identified 114 of 119 previously characterized *Achromobacter* isolates as *Achromobacter* spp., of which 48 (42%) were *A. xylosoxidans* (Table S2). The five Ac-Ax-negative isolates, which were incorrectly identified as *Achromobacter* sp. according to API 20 NE or ARDRA, were confirmed as *B. bronchiseptica*, *C. metallidurans*, *P. aeruginosa* or *S. maltophilia* based on 1.3 kb 16S rRNA gene sequencing. All five genus misclassifications were also confirmed by WGS (data not shown). Previous studies have demonstrated the poor performance of API 20 NE for *Achromobacter* spp. identification, with *Bordetella petrii*, *Bordetella trematum*, *Ralstonia pickettii*, *Alcaligenes faecalis*, *Comamonas testosteronii*, *Moraxella* sp., *Pasteurella* sp. and *Pseudomonas alcaligenes* being incorrectly classified as *Achromobacter* spp., or vice versa [22, 48–50]. Based on these collective findings, we do not recommend ARDRA or API 20 NE for *Achromobacter* identification due to relatively high false-positivity rates with other common pathogens.

A major component of highly robust microbial diagnostics development is ensuring correct species identification and locus specificity for the target taxa. The gold standard method for achieving high-quality target identification is large-scale comparative genomic analysis to identify species- and genus-level boundaries. As demonstrated by our phylogenomic analysis (Fig. 1), nearly a quarter of the 158 publicly deposited *Achromobacter* genomes were incorrectly speciated. Our study therefore demonstrates the critical importance of using large-scale comparative genomics for informed and accurate diagnostics development.

The Ac-Ax assay demonstrated good sensitivity and specificity for achromobacterial identification from polymicrobial specimens, with 4/33 (12%) adult CF sputa being Ac PCR-positive. This rate falls within the 3–30% *Achromobacter* prevalence rates reported in CF centres worldwide [9–11]. Of the four positive sputa, one patient, SCHI0014, was found to harbour a non-*A. xylosoxidans* achromobacterial infection (Table 3). When compared with culture, 2/5 Ac PCR-positive samples were also culture-positive; as expected, culture-positive specimens correlated with sputa containing the highest *Achromobacter* DNA load (Table 3), with very low-abundance samples failing to culture *Achromobacter*, either due to the lower sensitivity of culture-based methods or cell nonviability. The Ac-Ax duplex PCR results were consistent with metataxonomic sequencing, which identified 5/33 (15%) achromobacterial-positive sputa. The one Ac-Ax PCR-negative sputum sample, collected on day 6 of intravenous antibiotic treatment in patient SCHI0030, had a very low (~0.1%) proportion of achromobacterial rDNA gene reads that exceeded the lower Ac-Ax LoD threshold (~12 and ~1 genome equivalent(s) for Ac and Ax, respectively). Notably,

the Ac-Ax assay detected very low prevalence of *A. xylosoxidans* in the day 1 sputum sample from patient SCHI0030 (C_T=34.7 for both Ac and Ax assays), which corresponded with a similarly low (~0.4%) proportion of achromobacterial rRNA gene reads, indicating very low prevalence of this organism in this patient's airways at both time points. Taken together, we show that the Ac-Ax assay provides good performance on polymicrobial specimens, with the advantage of a considerable reduction in cost and turnaround time compared with metataxonomic sequencing or culture-based methods.

In conclusion, we have employed a large-scale comparative genomics approach to design a highly accurate duplex real-time PCR assay for the rapid, sensitive, specific, cost-effective and simultaneous detection of *Achromobacter* spp. and *A. xylosoxidans* from purified cultures and polymicrobial clinical specimens. Implementation of the Ac-Ax assay in the clinic will enable same-day diagnosis of these naturally drug-resistant organisms, providing the opportunity for targeted antimicrobial therapy and rapid treatment shifts in response to achromobacterial detection. Although beyond the scope of the current study, future work should compare Ac-Ax and VITEK MS results across a large isolate panel to determine VITEK MS accuracy among *Achromobacter* spp.

Funding information
This study was funded by the University of the Sunshine Coast and Advance Queensland [awards AQRF13016-17RD2 (D. S. S.) and AQIRF0362018 (E. P. P.)]. T. J. K. is the recipient of a National Health and Medical Research Council Early Career Fellowship (1088448).

Acknowledgements
We wish to thank Timothy Wells and Amy Pham (Translational Research Institute, Brisbane, Australia) for providing *Prevotella* and *Veillonella* DNA for this study, and Danielle Madden (University of the Sunshine Coast) for laboratory assistance.

Author contributions
Conceptualization: E. P. P. and D. S. S.; methodology and experimental design: E. P. P., V. S. A., T. A. F. and D.S.S.; sample collection: T. J. K., T-K. N. and S. C. B.; writing – original draft preparation: E. P. P. and V. S. A.; writing – review and editing: T. J. K., T. A. F., T-K. N., S. C. B. and D. S. S.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
This study was approved by The Prince Charles Hospital Human Research Ethics Committee (HREC/13/QPCH/127). Written informed consent was provided by the study participants.

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