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IncP-type plasmids carrying genes for antibiotic resistance or for aromatic compound degradation are prevalent in sequenced *Aromatoleum* and *Thauera* strains

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Abstract

Self-transferable plasmids of the incompatibility group P-1 (IncP-1) are considered important carriers of genes for antibiotic resistance and other adaptive functions. In the laboratory, these plasmids have a broad host range; however, little is known about their in situ host profile. In this study, we discovered that *Thauera aromatica* K172^T, a facultative denitrifying microorganism capable of degrading various aromatic compounds, contains a plasmid highly similar to the IncP-1 ε archetype pKJK5. The plasmid harbours multiple antibiotic resistance genes and is maintained in strain K172^T for at least 1000 generations without selection pressure from antibiotics. In a subsequent search, we found additional nine IncP-type plasmids in a total of 40 sequenced genomes of the closely related genera *Aromatoleum* and *Thauera*. Six of these plasmids form a novel IncP-1 subgroup designated θ, four of which carry genes for anaerobic or aerobic degradation of aromatic compounds. Pentanucleotide sequence analyses (*k*-mer profiling) indicated that *Aromatoleum* spp. and *Thauera* spp. are among the most suitable hosts for the θ plasmids. Our results highlight the importance of IncP-1 plasmids for the genetic adaptation of these common facultative denitrifying bacteria and provide novel insights into the in situ host profile of these plasmids.

INTRODUCTION

Members of the closely related genera *Aromatoleum* and *Thauera* (Betaproteobacteriales) are facultative denitrifying bacteria that degrade various aromatic and aliphatic compounds under anoxic or oxic conditions. They are commonly found in wastewater treatment plants (WWTPs) and anoxic sediments, from which 25 type strains and numerous additional isolates have been obtained (Gorny et al., 1992; Heider & Fuchs, 2005; Lajoie et al., 2000; Mechichi et al., 2002; Rabus et al., 2019; Rabus & Widdel, 1995; Song et al., 2000). Among these strains is *Thauera aromatica* K172^T (Anders et al., 1995; Tschech & Fuchs, 1987), which has been used to great advantage as model organism to study the anaerobic degradation of

aromatic compounds via the benzoyl-CoA pathway (Fuchs et al., 2011; Harwood et al., 1998).

Here, we sequenced the genome of strain K172 and discovered that it contains a plasmid of the incompatibility group IncP-1. Previously, one of the two plasmids present in *Aromatoleum aromaticum* EbN1^T was tentatively classified as an IncP-1 plasmid (Rabus et al., 2005). Members of this plasmid group are self-transmissible and have a broad host range with transconjugation to diverse Gram-negative and even Gram-positive bacteria (Macedo et al., 2022; Sen et al., 2011; Sota et al., 2007; Top & Springael, 2003). They carry the genes for conjugative DNA transfer (*tra*, *trb*) and initiation of DNA replication (*trfA*, *ssb*), regulation, partitioning, and stability (*incC*, *kfr*, *klc*, *kle*, *kor*, *par*) in highly conserved backbone regions. Based on the phylogeny of backbone genes, seven IncP-1 subgroups, α to η, have been identified so far (Bahl, Hansen, Goesmann, & Sørensen, 2007; Brown et al., 2013; Haines et al., 2006; Norberg et al., 2011; Pansegrau et al., 1994; Thorsted et al., 1998; Vedler et al., 2004). Besides serving as models for studying conjugative DNA transfer and replication, IncP-1-type plasmids have gained considerable prominence because they can harbour accessory regions containing genes for diverse adaptive functions such as resistance to antibiotics and aerobic degradation of aromatic compounds (Bahl, Hansen, Goesmann, & Sørensen, 2007; Pallares-Vega et al., 2021; Waters & Guiney, 1993). Together with their broad host range and comparably high conjugation rates, they therefore mediate the distribution of genetic information over vast phylogenetic distances and contribute to the adaptation of bacteria in natural and clinical environments (Klümper et al., 2015; Schlüter et al., 2007; Yano et al., 2013).

Apart from the identification in clinical isolates, many IncP-1 plasmids were retrieved by exogenous plasmid capturing from rhizospheres, soils, and organic substrates such as manure in Europe without knowledge of their in situ hosts (Bahl, Hansen, Goesmann, & Sørensen, 2007; Binh et al., 2008; Heuer et al., 2012; Jechalke et al., 2013; Shintani et al., 2020; Wolters et al., 2015). The implications for the prevalence of antibiotic resistance have prompted studies to elucidate the in situ and evolutionary host range of these plasmids. Sensitive conjugation assays with model IncP-1 plasmids revealed promiscuous conjugation with diverse members of soil and WWTP communities covering a wide phylogenetic distribution of up to 13 phyla (Klümper et al., 2015; Li et al., 2018, 2020; Macedo et al., 2022). The profiles of the plasmid recipients were distinct from the total communities. Various Gammaproteobacteria and Actinobacteria were apparently more permissive for plasmid uptake than other members of the communities, indicating that plasmid acquisition was not a stochastic process. Potential long-term evolutionary host ranges have been assessed via computing frequencies of oligonucleotides, the so-called *k*-mers, in chromosomal and plasmid DNA. Similar *k*-mer frequencies are interpreted as an amelioration of the plasmids' nucleotide sequence towards that of the chromosome during long-term residence in the host (Campbell et al., 1999; Lawrence & Ochman, 1997; Suzuki et al., 2010). Several Gammaproteobacteria, in particular those belonging to the order Betaproteobacteriales, have been identified as potential long-term hosts of IncP-1 plasmids based on *k*-mer profiling (Bahl, Hansen, & Sørensen, 2007; Li et al., 2018, 2020; Norberg et al., 2011; Suzuki et al., 2008; Yano et al., 2013). *Aromatoleum aromaticum* EbN1^T was implicated as one of the most matching host for the IncP-1 plasmid pB10 among the Betaproteobacteriales strains (Suzuki et al., 2008).

Based on these findings, we searched for plasmids in all publicly available genomes of strains of the genera *Aromatoleum*, *Thauera* and the closely related *Azoarcus* and *Zoogloea*. Our results suggest that IncP-1 plasmids are widely distributed in the genera *Aromatoleum* and *Thauera* as gene carriers for adaptive traits, including antibiotic resistance and aromatic compound degradation. Furthermore, we show that several genes for anaerobic degradation of aromatic compounds are located on plasmids of a novel IncP-1 subgroup, designated θ, for which the two genera may be long-term hosts according to *k*-mer profiling.

EXPERIMENTAL PROCEDURES

Bacterial strains and cultivation

Thauera aromatica K172^T (DSM 6984) was obtained from the DSMZ (Braunschweig, Germany), from our own culture collections, and from the collection of G. Fuchs (University of Freiburg, Germany). The strain had nowhere been cultured in medium containing an antimicrobial reagent prior to this study. All other strains were obtained from G. Fuchs, B. Schink (University of Konstanz, Germany), B. Philipp (University of Münster, Germany), and our collection. Strains of *T. aromatica*, *Ar. toluolicum* T^T, and *Ar. anaerobium* LuFRes1^T were cultured under anoxic conditions at 30°C in liquid mineral medium (DSMZ 586) with 5 mM nitrate as electron acceptor and either 1 mM benzoate, 1 mM resorcinol or 5 mM acetate as the sole carbon and electron source (Tschech & Fuchs, 1987). Bacterial growth was determined via measurement of the optical density at 600 nm. *Ar. buckelii* U120^T and *T. phenylacetica* B4P^T were grown under oxic conditions in a 1:10 dilution of trypticase soy medium, or full-strength Müller–Hinton at 28°C. When appropriate, antibiotics were added to the medium at the following final concentrations: tetracycline (10 μg/ml), erythromycin (200 μg/ml), sulfamethoxazole (60 μg/ml), ampicillin (0.01–5 μg/ml),

meropenem (1 μg/ml), imipenem (1 μg/ml), and ceftriaxone (25 and 50 μg/ml).

Conjugation assays

Conjugation was performed using *T. aromatica* K172^T as the donor, grown in DSMZ medium 586 containing 5 mM acetate, and the recipient strain grown in trypticase soy broth at one-tenth strength. Donor and recipient cultures were grown to cell densities of approximately 10^5 and 10^7 cells/ml, respectively, which were quantified using a Coulter counter (Beckman Coulter Life Sciences, Krefeld, Germany). Then, 200 μl of the donor culture and 20 μl of the recipient culture were mixed in a 1.5 ml Eppendorf tube and incubated overnight at 20°C either under oxic conditions or anoxically in a glove box (Coy Laboratory Products, Grass Lake, MI, USA). Subsequently, 50 μl aliquots of the conjugation assays were plated on one-tenth trypticase soy agar containing tetracycline and erythromycin. Strain K172^T grew very poorly on this medium. Up to five colonies of presumptive transconjugates from each agar plate were tested for the presence of pKJK172 in PCRs targeting *ereA*, *kleB*, *tetA*, and *traI* (Table S5).

Genome sequencing, assembly, annotation and genome mining

The genomic DNA sequenced in this research was extracted by the Blood and Tissue kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Whole-genome sequencing of *T. aromatica* K172^T was performed using 2 × 50 bp mate-pair reads (3–4 kb inserts) by Illumina HiSeq (Illumina, California, USA) and 454 pyrosequencing with a GS Junior sequencer (Roche Life Sciences, Penzberg, Germany). The three other *T. aromatica* strains (LG356, SP, and AR-1) were sequenced on an Illumina NexSeq550 (Illumina) using 150 bp pair-end reads. *Aromatoleum anaerobius* LuFRes1^T and *Ar. buckelii* U120^T were sequenced by Oxford Nanopore Technologies (ONT) (Oxford Nanopore Technologies, Oxford, UK) platforms for hybrid assembly with published Illumina reads (Raittz et al., 2021). Library preparation, read quality assessment and trimming, and assembly are described in detail in the Supplementary Information S1.

PCR primers used to circularize the plasmid sequences in *Ar. toluolicum* T^T and *Aromatoleum* sp. PA01 and to verify the assembly of the plasmid-containing contig (NZ_AMXF01000006) of *T. phenylacetica* B4P^T are provided in the Supplementary Information S1. PCR products were sequenced with the Sanger method (Eurofins).

Automated annotation was carried out via NCBI's Prokaryotic Genome Annotation Pipeline. Antibiotic resistance gene markers were annotated using ResFinder 2.1 (Zankari et al., 2012) and the Comprehensive Antibiotic Resistance Database (CARD) (Alcock et al., 2020). Prophages were searched for using PHASTER (Arndt et al., 2016) and refined by manual inspection. Genomic islands were identified by IslandViewer (Bertelli et al., 2017) and the whole-genome and plasmid alignments were carried out with MAUVE (Darling et al., 2010) and Easyfig v2.2.2 (Sullivan et al., 2011). Genomic graphs were generated by CGview (http://wishart.biology.ualberta.ca/cgview). The annotated chromosomes and plasmids from this research have been deposited at GenBank with accession numbers listed in the supplementary information S1.

The presence of plasmids in genomes of members of *Aromatoleum*, *Azoarcus*, *Thauera* and *Zoogloea* deposited in GenBank and JGI GOLD (Table S2) was manually queried for in the annotated sequences via BLASTn with the *tra* and *trb* gene clusters. In addition, we manually investigated all regions with no close homology to the chromosomes of *T. aromatica* K172^T or *Ar. aromaticum* EbN1^T as identified through MAUVE alignments.

Plasmid phylogeny and nucleotide signature analysis

The *traI* gene sequence was manually extracted from of 39 IncP-1 type plasmids deposited in GenBank and those identified in this study. The ClustalW alignment and maximum likelihood phylogenetic tree was constructed using MEGA7 (Kumar et al., 2016). The *k*-mer frequencies of the two backbone regions encompassing the *tra* and *trb* genes from 10 IncP-1 plasmids were individually compared with the ribosomal protein gene region of chromosomes of selected strains (Figure 6). To prevent *k*-mer frequency counting bias caused by different input sequence sizes, chimeric plasmid structures and horizontally acquired regions in the chromosomes, 14,200 bp-long nucleotide sequences from the plasmids' *tra* and *trb* regions as well as ribosomal protein regions from the chromosomes were used. Pentanucleotide (5-mer) frequencies were computed using Jellyfish (version 2.3.0) (Marçais & Kingsford, 2011). The pairwise similarities between the regions were then calculated by Kendall correlation, using the built-in R function cor(), from which a hierarchical clustering heatmap was computed and visualized with the pheatmap package in R.

RESULTS AND DISCUSSION

Genomic features of *T. aromatica* K172^T

Since strain K172^T is an important model organism for the study of anaerobic degradation of aromatic

compounds, we first focus on describing genomic features of this strain and then present our findings on plasmids across the genera *Aromatoleum* and *Thauera*. The complete genome of strain K172^T consists of one circular chromosome (3.7 Mb, 67.3% GC content) and one circular plasmid (53.8 kb, 60.8% GC content) (Figure 1). The plasmid is hereafter referred to as pKJK172. The label is derived from the name of the close relative pKJK5 (Bahl, Hansen, Goesmann, & Sørensen, 2007) and the strain name K172. The chromosome carries three presumably incomplete prophages, two integrative conjugative elements (ICE), and 12 additional clusters with genes of higher mobility. Other general genome features are summarized in Table S1. All genes previously identified for aromatic compound degradation in *T. aromatica* K172^T were found in the chromosome (Figure 1A, Table S2). In this study, the genes for degradation of *p*-cresol (*pch*) and indoleacetate (*iaa*), known growth substrates of strain K172^T (Anders et al., 1995), were also detected in the chromosome, as well as genes for phenyl- or indolepyruvate degradation (*ior*). Furthermore, we found the full set of genes predicted to be involved in the anaerobic degradation of toluene via benzylsuccinate in other denitrifying bacteria also in strain K172^T (Achong et al., 2001; Coschigano 2000; Kube et al., 2004; Meyer-Cifuentes et al., 2020).

For several denitrifying bacteria including *Aromatoleum* sp. CIB and *Magnetospirillum* sp. 15-1, there is sequence evidence that anaerobic toluene degradation is a horizontally acquired trait (Blazquez et al., 2018; Heider et al., 2016; Meyer-Cifuentes et al., 2020). There are three lines of evidence that the anaerobic toluene degradation gene cluster (*bss*, *bbs*, and *tdi* genes) was also horizontally acquired in *T. aromatica* K172^T. First, the cluster is only present in this strain and in *T. chlorobenzoica* 3CB-1^T among the genome-sequenced *Thauera* strains, which includes the three *T. aromatica* strains AR-1, LG356, and SP sequenced in this study (see below). Overall, the synteny and shared gene content of the four *T. aromatica* genomes are high (Figure S1), and all other genes of strain K172^T involved in aromatic compound degradation are present in the other three strains, except for the absence of the *ppc* genes for phenol degradation in strain AR-1. Second, genes encoding a type II toxin/antitoxin system are located between the *bss* and *bbs* genes. Toxin/antitoxin systems are often associated with mobile genetic elements, including low-copy plasmids where they were first discovered (Kedzierska et al., 2007; Ramisetty & Santhosh, 2017). Third, the *tdi* and *bss* genes in strains K172^T and 3CB-1^T are 94.9% similar, whereas the *bbs* genes are 99.9% similar. Previously, different evolutionary histories of the *bss* and *bbs* gene clusters in *Aromatoleum* sp. CIB were proposed based on their different GC% content (Blazquez et al., 2018). Therefore, it is parsimonious to assume that *tdi*, *bss*, and *bbs* genes were acquired horizontally by strains K172^T and 3CB-1^T or their close ancestors after the split into species and strain lineages.

Likewise, the presence of the genes for anaerobic degradation of α-resorcylate via the hydroxyhydroquinone (HHQ) pathway in strains AR-1 and 3CB-1^T, the genes for ethylbenzene degradation in *Ar. aromatoleum* EbN1^T, and the genes for *p*-cymene degradation in *Ar. aromatoleum* pCyN1 appear to be due to horizontal transfer (Becker et al., 2022; Molina-Fuentes et al., 2015; Pacheco-Sanchez et al., 2019).

Features of plasmid pKJK172

The backbone of pKJK172 (Figure 1B), totalling 40.6 kb, is identical to that of the IncP-1 ε archetype pKJK5 isolated from manured soil without knowledge of its in situ host (Bahl, Hansen, Goesmann, & Sørensen, 2007; Bahl, Hansen, & Sørensen, 2007). Plasmid pKJK172 belongs to the ε-I type as its accessory region comprises a Tn*402*-like transposon with the tetracycline resistance genes *tetRA*, an *eamA* family multidrug transporter, IS*1326* insertion sequences, and a clinical class I integron derivative. The region also contains a putative post-segregational killing system bordered by ISPa17 insertion sequences (Li et al., 2020). Other than the integron derivative, the accessory region in pKJK172 has 99.97% nucleotide sequence identity with that in pKJK5. The integron derivative contains a fragment of a class I integron (confirmed by Sanger sequencing), the 5′-fragment of an aminoglycoside acetyltransferase gene, the erythromycin esterase gene *ere*(A), and the resistance genes against quaternary ammonium compounds (*qacE*Δ1) and sulfonamides (*sul1*) in the 3′-conserved segment of the integron. The class 1 integron in pKJK5 carries gene cassettes for an aminoglycoside adenyltransferase and for trimethoprim resistance (Bahl, Hansen, Goesmann, & Sørensen, 2007). We confirmed that strain K172^T exhibits resistance to tetracycline (10 μg/ml), erythromycin (200 μg/ml), and sulfamethoxazole (60 μg/ml). *Thauera aromatica* strains AR-1 and LG356 do not carry the plasmid and are susceptible to these antimicrobials.

Plasmid pKJK172 could be transferred in conjugation assays from strain K172^T to *Sphingobium yanoikuyae* B1 (Alphaproteobacteria), *Escherichia coli* K12, *Paraburkholderia sartisoli* RP007^T, and *Pseudomonas putida* KT2440 (all Gammaproteobacteria), *Weizmannia coagulans* NRS609^T (Firmicutes), and the two Gram-positive Actinobacteria, *Mycobacterium phlei* 35 and *Streptomyces griseus* 18-16^T, thus covering the broad phylogenetic host range of the archetype pKJK5 (Klümper et al., 2015; Li et al., 2020). The transfer frequency was approximately 10^{-5} with all recipient

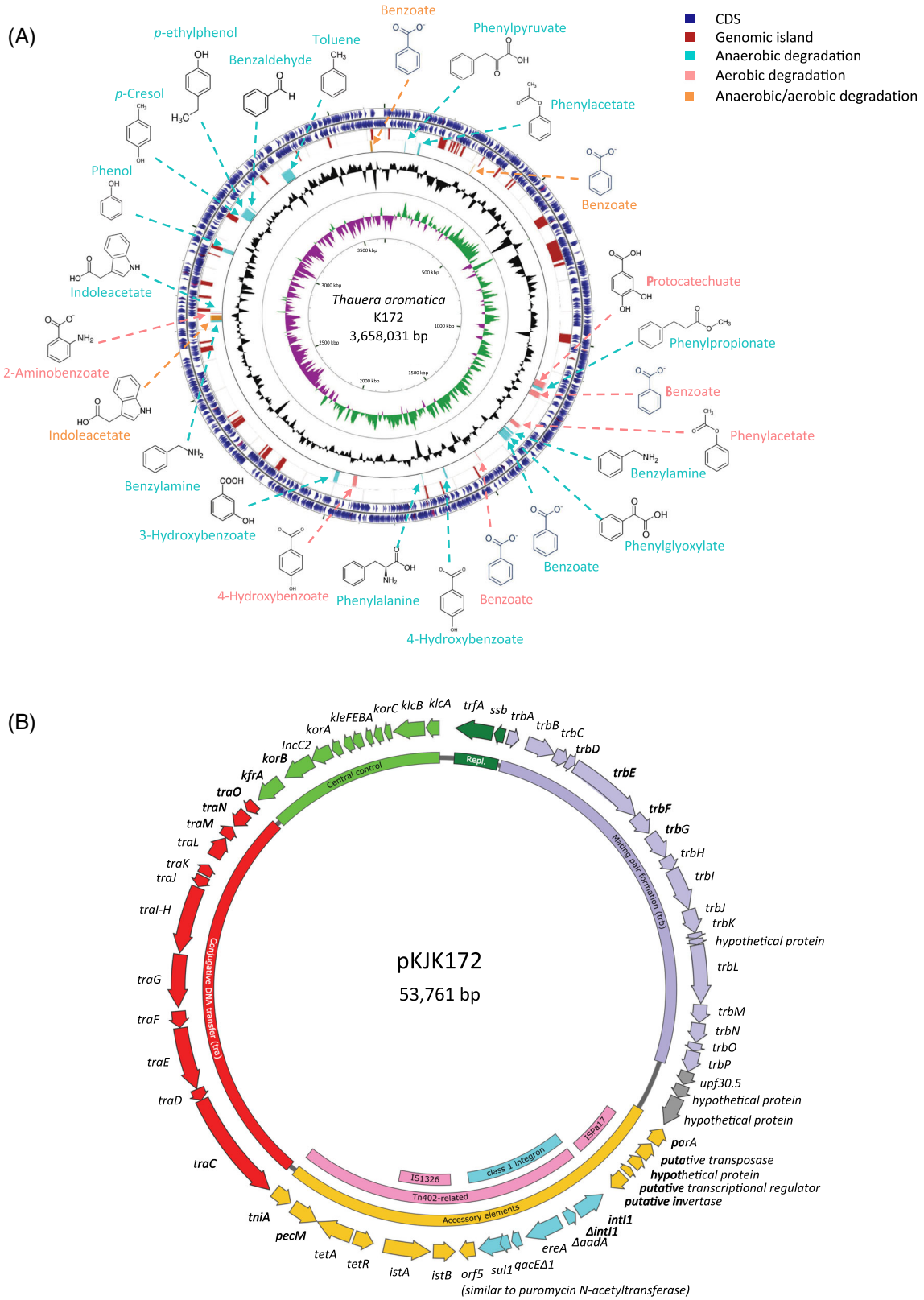

FIGURE 1 Genome of *Thauera aromatica* K172. (A) Features in the chromosome are shown by circles from the outside to the inside as follows: predicted coding sequences (navy blue); genomic islands (red); genes involved in degradation of aromatic compounds (blue: anaerobic degradation, pink: aerobic degradation); GC content (black); and GC skew (green: positive, purple: negative). (B) Organization of the IncP-1 ε plasmid, pKJK172. Shown are the backbone region for the replication module (dark green), mating pair formation (purple), conjugative DNA transfer (red), central control region (brown), and the accessory region (yellow) containing a class 1 integron derivative (turquois).

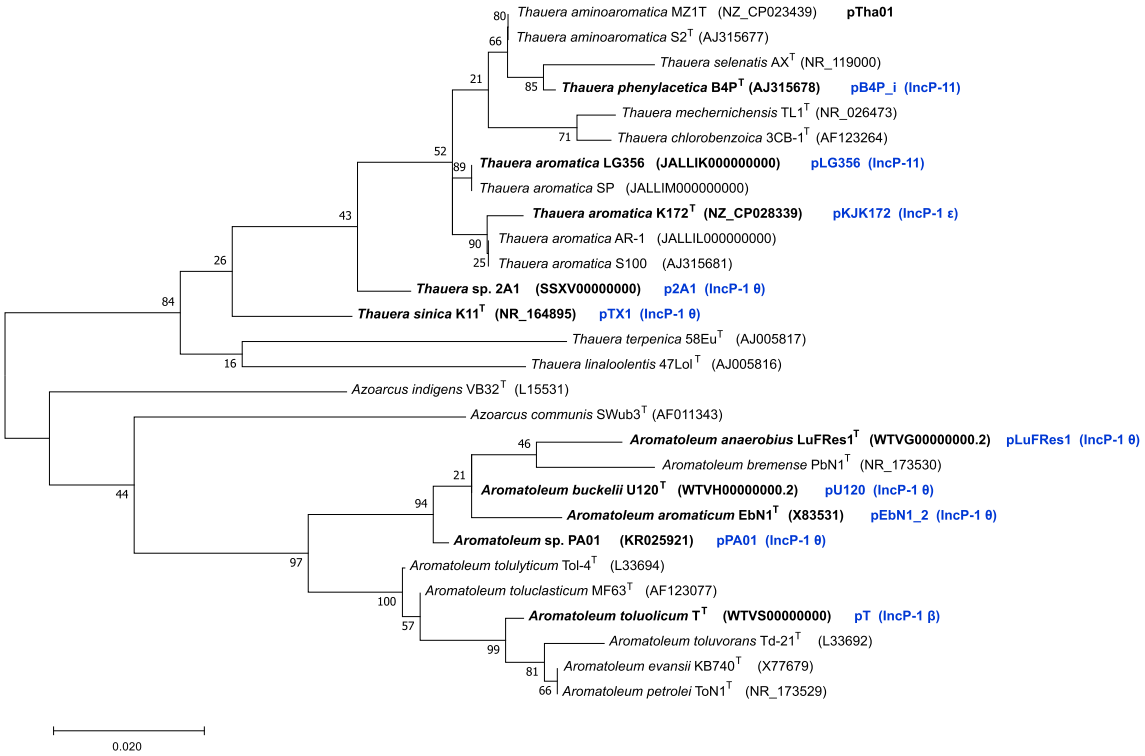

FIGURE 2 Phylogeny of selected *Aromatoleum* spp. and *Thauera* spp. based on 16S rRNA gene sequences. GenBank accession number are in parenthesis. The strains that contain IncP-type plasmids (blue) are shown in bold. The maximum-likelihood tree was constructed using MEGA version 7.0 (Kumar et al., 2016), with the scale bar corresponding to the nucleotide substitution rate. Bootstrap values of 400 replications are given in percentage (%) for each branch.

strains, which is about one order of magnitude lower than with *E. coli* and *P. putida* as donors of pKJK5 (Li et al., 2018). The lower frequency with *T. aromatica* K172^T might be explained by sub-optimal conditions of the conjugation assay. Plasmid pKJK5 is only poorly transferred in liquid medium, probably due to the short and rigid pili assembled from its Tra proteins (Bahl, Hansen, & Sørensen, 2007; Bradley, 1984). Further, strain K172^T grows poorly on agar plates and has low fitness on solid media.

Plasmid maintenance was evaluated with subcultures of *T. aromatica* K172^T from four different collections where the strain had never been cultured in the presence of antimicrobials. The subcultures had been propagated up to 200 times with approximately 5–10 duplications per cultivation. In each subculture, PCR assays were positive for all tested genes of pKJK172 (*ereA*, *kleB*, *tetA, tfrA*, *traI*, and *trbD*). Thus, pKJK172 was maintained in strain K172^T for at least 1000 generations without selective pressure by antimicrobials. Stable maintenance of IncP-1 plasmids including pKJK5 in the absence of selective pressure has been observed previously (De Gelder et al., 2007; Heuer et al., 2007, 2010; Sota et al., 2010). There are at least three potential mechanisms that could individually or collectively prevent the disappearance of pKJK172 from the population of strain K172^T. First, the predicted killing system on the plasmid might be functional. Second, the conjugation rate, although low, might be sufficiently high to counteract segregational losses in individual cells by re-conjugation, as has been described for pKJK5 (Bahl, Hansen, Goesmann, & Sørensen, 2007). Third, the intrinsic fitness cost of the plasmid to the host could be negligible. There appear to be host-specific factors that affect IncP-1 plasmid persistence in a population (Li et al., 2020) which might differ between strains of the same species (Heuer et al., 2010). The reason for the variability in persistence between replication hosts is still unclear.

Plasmids in other members of *Thauera* and *Aromatoleum*

To search for plasmids in other members of the genus *Thauera* and the closely related genera *Aromatoleum*, *Azoarcus* and *Zoogloea*, we generated draft genome sequences of the three *T. aromatica* strains AR-1, LG356, and SP and analysed all publicly available genomes of strains affiliated with the four genera (Table S3). In this process, we verified and improved the already available genome assemblies of *Ar. anaerobius* LuFRes1^T, *Ar. buckelii* U120^T, *Ar. toluolicum* T^T, *Aromatoleum* sp. PA01, *T. phenylacetica* B4P^T, and *Thauera* sp. 2A1.

TABLE 1 Plasmids in sequenced *Aromatoleum* spp. and *Thauera* spp.

Host	Plasmid	IncP subgroup	Size (bp)	Genetic features in accessory region
Ar. aromaticum EbN1	pEbN1_2	1-θ	223,670	Anaerobic degradation of *o*-phthalate
Aromatoleum sp. PA01	pPA01	1-θ	226,855	Anaerobic degradation of *o*-phthalate
Ar. buckelii U120	pU120	1-θ	334,342	Anaerobic degradation of *o*-phthalate, *p*-cresol; aerobic degradation of phenyl acetate
Ar. anaerobius LuFRes1	pLuFRes1	1-θ	115,562	Anaerobic degradation of resorcinol
Ar. toluolicum T	pT	1-β[a]	92,504	Mercury resistance (*mer* genes)
T. aromatica K172	pKJK172	1-ε	53,761	Antimicrobial resistance: erythromycin (*ereA*), sulfonamides (*sul1*), quaternary ammonium compounds (*qacE*Δ1), tetracycline (*tetA*)
T. aromatica LG356	pLG356	11	35,470	Molybdenum ABC transporter (*mod*)
T. phenylacetica B4P	pB4P_i[b]	11	36,335	Antimicrobial resistance: β-lactams (*bla*OXA205), aminoglycosides (*aad*A4), sulfonamides (*sul1*), quaternary ammonium compounds (*qacE*Δ1)
T. sinica K11	pTX1	1-θ	140,963	Heavy metal resistance (two efflux pumps), arsenic resistance (*arsB*-like)
Thauera sp. 2A1	p2A1	1-θ	175,000	Mercury resistance (*mer*)
T. aminoaromatica MZ1T	pTha01	none	78,374	Heavy metal resistance (efflux pump, translocating P-type ATPase)

[a]Chimeric plasmid.
[b]Plasmid integrated into the chromosome.

Together with the published plasmids pEbN1_2 in *Ar. aromaticum* EbN1^T (Rabus et al., 2005) and pTX1 in *T. sinica* K11^T (GenBank accession number CP023440), we found a total of 11 plasmids in the 40 sequenced genomes of *Thauera* and *Aromatoleum* strains (Figure 2; Table 1). Three of the 26 sequenced *Thauera* strains carry an IncP-1 type plasmid, two strains harbour an IncP-11 plasmid, and *T. aminoaromatica* MZ1T carries an unclassified plasmid that is not considered further in this study (Jiang et al., 2012). Among the 14 *Aromatoleum* genomes, four contain an IncP-1 type plasmid and one a chimeric plasmid with an IncP-1 β fragment. In contrast, no IncP type plasmid was detected in any of the 18 *Azoarcus* genomes or in the seven *Zoogloea* genome assemblies analysed. A replicon of 738 kb in *Azoarcus* sp. KH32C (Nishizawa et al., 2012) was the only plasmid found in these two genera (although strain KH32C may need to be reclassified as *Aromatoleum* sp. according to whole-genome comparisons; Raittz et al., 2021). A search in the plasmid database PLSDB (Galata et al., 2019) revealed no other genus with a similarly high prevalence of IncP-type plasmids among the sequenced representatives as *Aromatoleum* and *Thauera*.

To further classify the IncP plasmids from the *Aromatoleum*/*Thauera* clade, we constructed a phylogenetic tree based on the *traI* gene, which encodes a conjugative relaxase and has been previously used as a phylogenetic marker (Garcillan-Barcia et al., 2009; Jechalke et al., 2013; Sen et al., 2011). The alternative phylogenetic marker *trfA* is not sufficiently conserved in all plasmids of this study and was therefore not used. The phylogenetic tree shown in Figure 3 includes the seven previously named IncP-1 subgroups α, β, γ, δ, ε, ζ, and η. The more distantly related IncP-11 plasmids are not included in the tree (Bonnin et al., 2018). With the exception of pKJK172, all complete IncP-1 type plasmids from the *Aromatoleum*/*Thauera* clade form a novel subgroup to which we assign the Greek letter θ. The θ subgroup contains the plasmids from *Ar. anaerobius* LuFRes1^T, *Ar. aromaticum* EbN1^T, *Ar. buckelii* U120^T, *Aromatoleum* sp. PA01, *T. sinica* K11^T and *Thauera* sp. 2A1. The four *Aromatoleum* strains were isolated from mud and WWTPs located in Germany, whereas the two *Thauera* strains were isolated from WWTPs in China (Table S2). Figures 2 and 3 show that the topologies of the phylogenetic trees of the *traI* and the 16S rRNA genes are not entirely congruent. The plasmids pTX1 and p2A1 were found in *Thauera* spp. but are more closely related to plasmids from *Aromatoleum* spp. We think this lack of congruency is due to plasmid transfer between hosts rather than miscalculated tree topologies.

Features of the IncP-1 θ plasmids

The backbone regions of the IncP-1 θ plasmids have overall around 77%–82% nt sequence similarity to the backbone regions of members of other IncP-1 subgroups. The *tra* and *trb* regions have high synteny to other IncP-1 plasmids. However, there are differences in the regions for initiation of replication (*trfA-ssb*), and central control (genes for regulation, partitioning, and

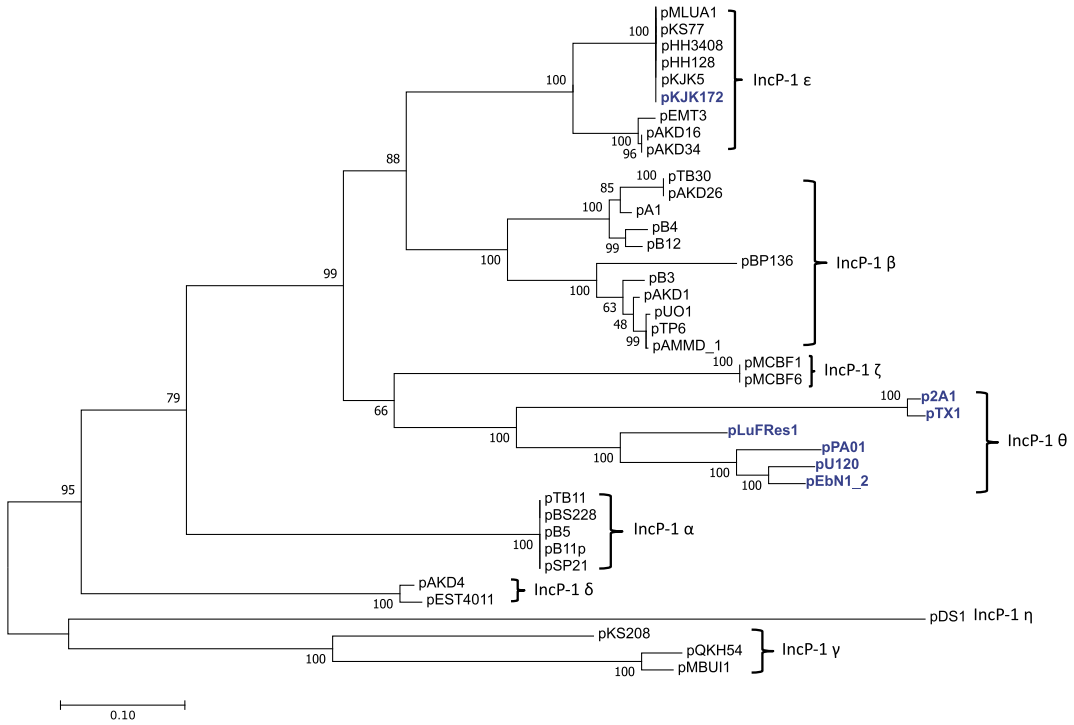

FIGURE 3 Phylogeny of IncP-1 plasmids based on *traI*. The Maximum-likelihood tree displays eight IncP-1 subgroups, including the novel θ subgroup identified in this study. The plasmids found in *Aromatoleum* spp. and *Thauera* spp. are shown in bold. The scale bar corresponds to the nucleotide substitution rate. Bootstrap values of 400 replications are given in percentage (%) for each branch. The tree was constructed using MEGA version 7.0.

stability). A complete copy of the *trfA* gene was found only in pLuFRes1, whereas this gene appears to be truncated in pEbN2 and was not found in the other IncP-1 θ plasmids (Figure S2). Moreover, homologues to the *ssb* were not found in any of the θ plasmids. The functions of these genes are of critical importance, and presumably these genes have been replaced non-homologously. In other IncP-1 plasmids, that region has been described as hot spot for the insertion of transposable elements (Sota et al., 2007). In the θ plasmids, the region may have been replaced during such as transposition event. Furthermore, *kfrA* was clearly identified only in pLuFRes1 but not in the other θ plasmids, and at the locus of *kleF* is a small gene that is predicted to code for a hypothetical protein with homologues found in various Gammaproteobacteria.

We now focus on the accessory regions of the IncP-1 θ plasmids. *Aromatoleum anaerobius* LuFRes1^T has been used as a model organism to study the anaerobic degradation of resorcinol (1,3-dihydroxybenzene) (Darley et al., 2007, 2020; Gorny et al., 1992; Philipp & Schink, 1998). The 115.6 kb-long plasmid pLuFRes1 contains the previously identified gene cluster for anaerobic degradation of resorcinol via the HHQ pathway in one of its two accessory regions (Figure 4B). Compared to the gene cluster deposited in GenBank (accession number EF078692), a group of 11 genes is inverted in pLuFRes1. Read mapping ascertained that this is a true inversion and not an assembly error. Upstream, the cluster is bordering to the *trb* region with genes coding for an IS*630* family transposase and a putative toxin/antitoxin system. At the downstream side, genes for an ABC transporter are located. These genes have homologues (>66% nt sequence identity) in the chromosome of several *Variovorax* spp. such as strain PAMC28562 (accession number CP060296). In that strain, the ABC transporter genes are adjacent to a gene cluster for aerobic degradation of HHQ, indicating that the transporter is involved in the uptake of hydroxylated benzenes. The other accessory region contains 17 transposase/recombinase/integrase genes that surround genes for enzymes of central and peripheral carbon metabolism present also in pPA01 and pU120 (see next paragraph). The regions are reciprocal best BLASTn hits with sections in the chromosomes of *Ar. aromaticum* EbN1^T and *Ar. bremense* PbN1^T (92%–99.8% identity; Weiten et al., 2021), suggesting a common evolutionary history of the plasmids in the genus *Aromatoleum*.

The backbones of the plasmids pEbN1_2, pPA01, and pU120 are more similar to each other than to any other sequenced plasmids in GenBank based on reciprocal best BLASTn results (Figure 4A). Plasmid pEbN1_2 (223.7 kb) from *Ar. aromaticum* EbN1^T was previously identified and described (Rabus et al.,

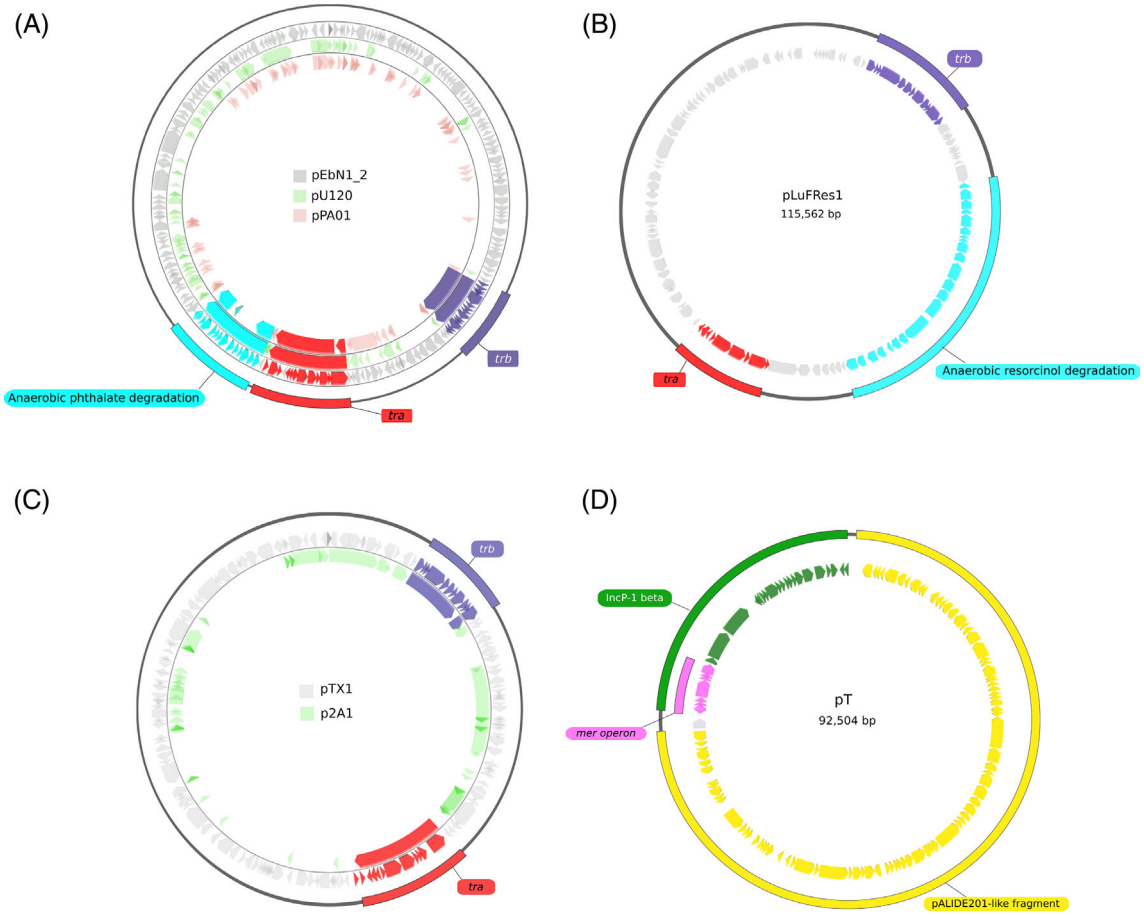

FIGURE 4 Maps of six novel IncP-1θ plasmids and the chimera plasmid pT from the *Aromatoleum*/*Thauera* clade with the gene regions for mating pair formation (purple) and conjugative DNA transfer (red). Plots were generated with CGview based on a BLASTn analysis. (A) Comparison of pEbN1_2 (outer ring), pU120 (middle ring, common genes in green) and pPA01 (inner ring, common genes in rose), which carry the gene cluster for anaerobic degradation of *o*-phthalate (turquois). (B) pLuFRes1 with the gene cluster for anaerobic degradation of resorcinol (turquois). (C) Comparison of pTX1 (outer ring) and p2A1 (inner ring, common genes in green). (D) Map of the two chimeric plasmid pT, apparently derived from an IncP-1 β plasmid (dark green) and a pALIDE201-like plasmid (yellow).

2005). Plasmid pPA01 (226.9 kb) was present as one of four linear contigs in the original genome assembly of *Aromatoleum* sp. PA01 based on PacBio long-read sequencing (Junghare et al., 2015). We noticed that the ends of the contig overlapped over a 7 kb stretch and generated a circular assembly by read mapping, PCR and Sanger sequencing. Plasmid pU120 (334 kb) from *Ar. buckelii* U120^T was assembled from published Illumina reads (Raittz et al., 2021) and Oxford Nanopore reads obtained in this study. The larger size of pU120 is predominantly due to an intact prophage of 41.3 kb, a putative prophage fragment of 10 kb, and a chemotaxis gene cluster (17.1 kb) most similar (around 70% nt sequence identity) to homologues in various other members of the *Rhodocyclaceae*. The three plasmids harbour various gene clusters for degradation of aromatic compounds. Adjacent to the *tra* region of all three plasmids is the recently identified gene cluster for anaerobic degradation of *ortho*-phthalate (Ebenau-Jehle et al., 2017). The cluster is also present in the chromosome of *T. chlorobenzoica* 3CB-1^T. In addition, plasmid pPA01 contains the *paa* gene cluster for aerobic catabolism of phenylacetate (Teufel et al., 2010). In plasmid pU120, we found the *pch* genes for degradation of *p*-cresol and, 5 kb upstream, a cluster of 15 genes involved in aerobic degradation of aromatic compounds, possibly via the homogentisate pathway. A gene coding for a class III estradiol aromatic ring-opening dioxygenase is not included in the gene cluster but is located on the chromosome of strain U120^T. Antibiotic resistance genes were not found in pEbN1_2, pPA01, and pU120.

Plasmid pTX1 (141 kb) from *T. sinica* K11^T was previously published in GenBank without classification (accession number CP023440), whereas plasmid p2A1 (175 kb) from *Thauera* sp. 2A1 was found in this study by improving the original genome assembly (Wang et al., 2020). Both plasmids share homologous regions totalling 64.3 kb with an average 99% sequence identity (Figure 4C), and which were reciprocal best

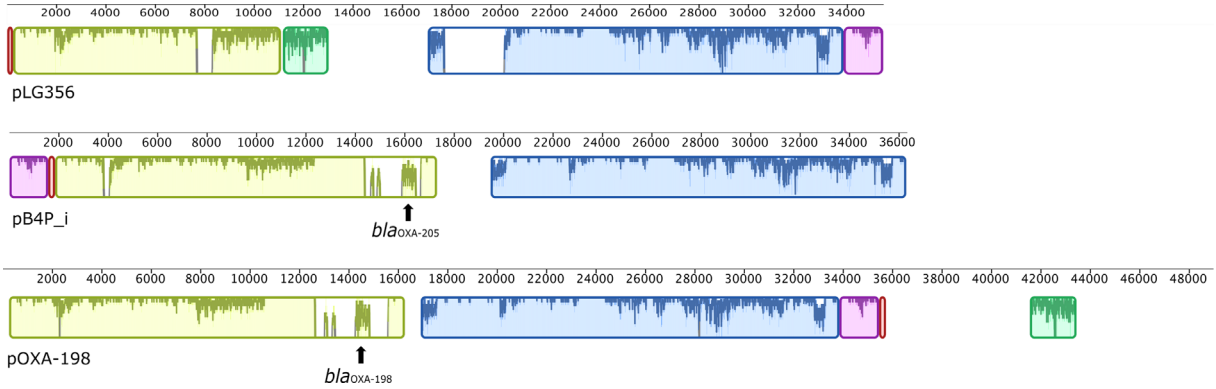

FIGURE 5 Alignment of pLG356, pB4P_i and the IncP-11 plasmid pOXA-198 (accession number NZ_MG958650) with Mauve (Darling et al., 2010). Absolute nucelotide postions are indicated by horizontal bars. The height of the coloured segments indicates the similarity in percent between the plasmids, and the white gaps represent non-homologous regions. The positions of genes coding for OXA-type beta lactamases in pB4P_i and pOXA-198 are indicated by arrows.

BLASTn hits to each other. Almost all of the accessory regions are homologous to chromosomal segments of various Betaproteobacteriales including *Ar. bremense* PbN1^T, *T. sinica* K11^T, *T. aminoaromatica* MZ1T, and *Azoarcus* sp. DD4. Plasmid pTX1 contains genes conferring resistance to heavy metals and arsenic, while p2A1 harbours a *mer* operon for resistance to mercury. No genes for antibiotic resistance and aromatic compound degradation were found in the two plasmids. Plasmids pTX1 and p2A1 carry 10 and 12 integrase/transposase/recombinase genes, respectively, indicating dynamic structures of these replicons.

We carried out a BLASTn search in GenBank with *traI* as query sequence and found three metagenome-assembled genomes (MAGs) with contigs displaying high similarity to segments from IncP-1 θ type plasmids, especially to pTX1 (Figure S3). With two of the MAGs, the strain with the best-matching assembly is *T. sinica* K11^T, and for the third MAG it is *T. aminoaromatica*. The first two MAGs were obtained from activated sludge from Shenzen, China (GenBank accession numbers GCA_018240785.1, GCA_018240845.1), whereas the third MAG was from a water purification facility in Orange County, CA, USA (Stamps & Spear, 2020). Because the genomes were assembled from complex environmental samples using short sequence reads, it is not certain that the three *Thauera*-like MAGs indeed contain IncP-1 θ type plasmids. Yet, the results are noteworthy since the plasmid-like contigs were binned together with the chromosomal *Thauera*-like contigs based on having the most similar *k*-mer profiles in the respective metagenome.

Features of the chimeric plasmid pT

Ar. toluolicum T^T was isolated from a laboratory-scale biodegradation column that had received aquifer inoculum from the Glatt river in Switzerland (Dolfing et al., 1990). Plasmid pT is a 92.5 kb-large chimera of an IncP-1 β plasmid and a conjugative plasmid without type-affiliation according to PlasmidFinder and pMLST analysis (Carattoli et al., 2014), with a fragment of a Tn*3*-family transposon in between (Figure 4D). The IncP-1 β part of pT is 22.4 kb in size and spans from a truncated *traL* on one side via the regulatory and stability region to a Tn*501*-like transposon with mercury resistance genes (*mer*) at the other side. The remainder of pT except the Tn*3*-family transposon fragment is 99.5% identical with perfect synteny to the 75 kb-large pALIDE201 from *Alicycliphilus denitrificans* K601^T (Betaproteobacteriales) isolated from the WWTP of Konstanz, Germany (Dangel et al., 1988; Oosterkamp et al., 2011). No genes for degradation of aromatic compounds or antibiotic resistance were found on this plasmid. Overall, the chimeric nature of pT accentuates that recombination of backbone regions can be important in plasmid evolution (Norberg et al., 2011).

Features of the IncP-11 type plasmids

The two novel plasmids, pB4P and pLG356, are of the IncP-11 type, which was first introduced for pOXA-198 found in clinical *P. aeruginosa* isolates and to which they are very similar (Figure 5) (Bonnin et al., 2018). Based on searches in PLSDB and GenBank, other known hosts of IncP-11 plasmids most frequently belong to the Gammaproteobacteria, including *Pseudomonas*, *Aeromonas*, and various *Enterobacteriaceae*. The hosts *T. phenylacetica* B4P^T and *T. aromatica* LG356 were isolated from the same WWTP in Ulm, Germany (Mechichi et al., 2002). Plasmid pB4P has a size of 36.3 kb and is integrated into the chromosome of strain B4P. Integration occurred most likely via the split *repA* gene, with the 5′ and 3′ fragments of the gene located at either end of the inserted plasmid. Sequence alignment with *repA* homologues revealed

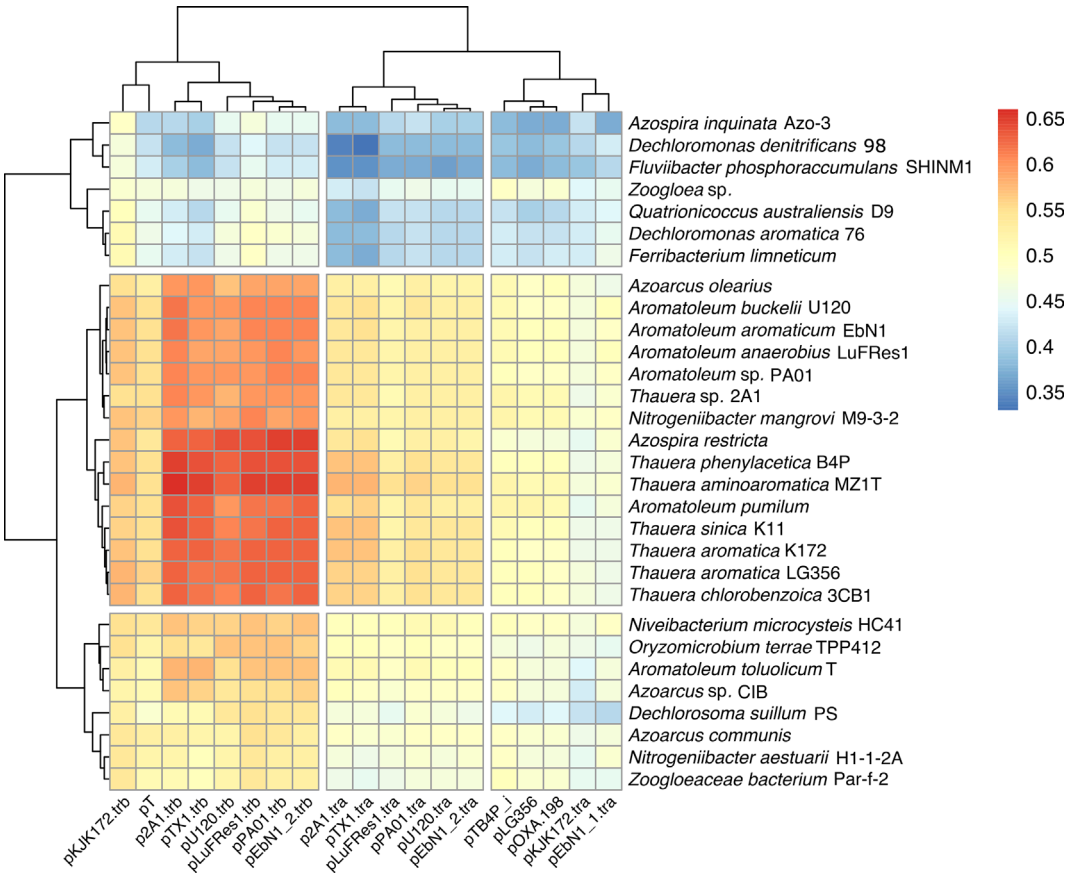

FIGURE 6 Comparative analysis of 5-mer frequency profiles of the ribosomal protein gene regions of sequenced members of the *Rhodocyclaceae*, 14.2 kb fragment from the three IncP-11 plasmids, and the *tra* and *trb* gene regions from seven IncP plasmids. The hierarchical clustering heatmap is based on calculated Kendall correlations of pairwise similarities. The scale bar represents Kendall's τ coefficient.

that the split position is mutated (confirmed by Sanger sequencing), meaning that without back-mutation the plasmid could not become circular once excised from the chromosome. Plasmid pB4P contains a novel clinical class 1 integron variety with a partially deleted *intI1*, the β-lactam resistance gene $bla_{\text{OXA-205}}$ and the aminoglycoside resistance gene *aadA*4. The $bla_{\text{OXA-205}}$ gene has 99.6% nt identity to a homologue in a clinical *P. aeruginosa* isolate (Krasauskas et al., 2015). It codes for a narrow-spectrum β-lactamase that was heterologously expressed in *E. coli*, purified, and characterized. The deduced sequences of the β-lactamases in strain B4P and the *P. aeruginosa* isolate differ by one amino acid residue (P230S). Although $bla_{\text{OXA-205}}$ conferred resistant to ampicillin to the *E. coli* expression host (minimum inhibitory concentration of 512 μg/ml), strain B4P^{T} is susceptible to that β-lactam (MIC of 0.01 μg/ml) (this study). Whether the amino acid substitution in the protein from strain B4P is the reason for the susceptibility of this strain is not known.

Plasmid pLG356 is only 20 bp larger than pB4P and has 92.9% sequence identity to it. In place of the class I integron, pLG356 contains a transposition module alongside genes for a predicted molybdenum ABC transporter and a transcriptional regulator. The entire segment of 8.4 kb is 99.99% identical to two IncFII-like plasmids in *Enterobacter* sp. E76 (GenBank accession number CP042500) and *Citrobacter* sp. R56 (CP069160), respectively. All sequenced *T. aromatica* strains carry an operon for a high-affinity molybdenum ABC transporter in their chromosome that is probably under the control of a ModE family transcriptional regulator. It is not apparent that strain LG356 would benefit from the additional molybdenum ABC transporter on pLG356, suggesting that this plasmid is a genetic parasite for its host.

Host profiling based on pentanucleotide signatures

Finally, we sought to refine previous results on the bioinformatically predicted host range of IncP-1 plasmids, where *k*-mer profiling was used and several families from the Betaproteobacteriales, including *Rhodocyclaceae*, were identified as top host candidates (Norberg

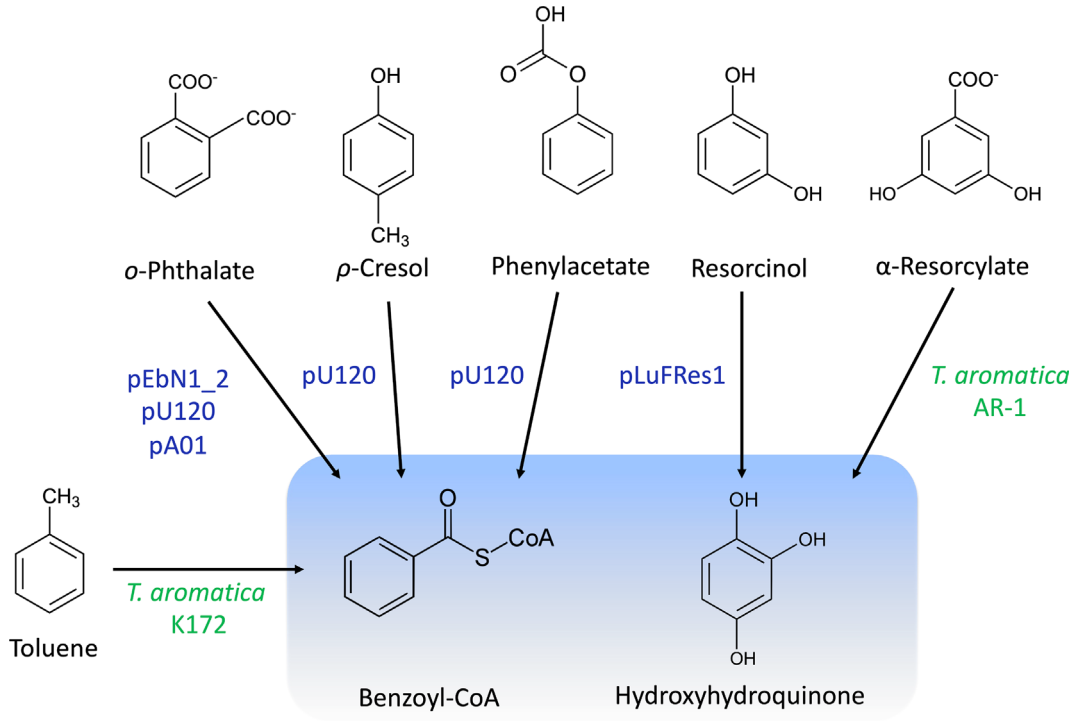

FIGURE 7 Overview of the ability to degrade aromatic compounds in *Aromatoleum* spp. and strains of *Thauera* aromatica via the key intermediates benzoyl-CoA and hydroxyhydroquinone due to horizontally acquired genes in the chromosome (green) or in IncP-1 plasmids (blue).

et al., 2011; Suzuki et al., 2008). To this end, we calculated the frequencies of penta-oligonucleotide, that is, the same *k*-mer length as previously chosen (Norberg et al., 2011), in equal-sized segments of the *tra* and *trb* regions of the IncP-1 and conserved portions of the IncP-11 plasmids from this study, and in the ribosomal protein gene region of selected 30 members of the *Rhodocyclaceae* (Figure 6). These strains were selected because they have a high degree of completion of the sequenced genomes.

The *tra* and *trb* regions had different correlations in their *k*-mer profiles with the ribosomal protein gene segment of a given strain, reflecting the mosaic structure of IncP-1 plasmids and indicating different evolutionary histories of the two regions. Moreover, the results suggest that among the *Rhodocyclaceae*, members of the genera *Aromatoleum* and *Thauera* are particularly suitable or even long-term hosts for IncP-1 θ plasmids. The *k*-mer profile of pKJK172 was not quite as similar to that of *T. aromatica* K172^T as were the profiles of the θ subgroup plasmids to those of their hosts, indicating that pKJK172 has not been ameliorated as much to its host.

CONCLUSION

This study provides information on genomic content and gene acquisition for members of the closely related denitrifying genera *Aromatoleum* and *Thauera*. We present a catalogue of genes involved in the anaerobic and aerobic degradation of monoaromatic compounds in the model organism *T. aromatica* K172^T. Furthermore, we show that gene clusters for the degradation of the aromatic compounds toluene, *p*-cresol, *o*-phthalate, phenylacetate, resorcinol and α-resorcylate were acquired horizontally by strain K172^T and other members of the genus *Thauera* as well as *Aromatoleum* (Figure 7). Important mobile genetic elements for the horizontal acquisitions of these genes are broad host plasmids of the IncP-1 type, in particular of the novel subgroup θ identified in this study. We also present two examples for IncP-type plasmids as carriers of antibiotic resistance genes in *Thauera* spp. The first example is an IncP-1 ε plasmid in strain K172^T that is nearly identical to the archetype pKJK5, which has been used extensively as a model to study conjugation and replication of broad host range plasmids. The second example is a chromosomally integrated plasmid harbouring a β-lactamase in *T. phenylacetica* B4P^T. *Thauera* spp. as well as *Aromatoleum* spp. are common members of the microbial community of WWTPs and other habitats where the spread of antibiotic resistance is of concern and where these microbes may be an important reservoir of respective genes. Both our empirical findings and the in silico analysis of *k*-mer similarity support that the two genera are top candidates for acquisition and long-term replication of IncP-1 plasmids.

AUTHOR CONTRIBUTIONS

Conceptualization: HYL and JAM; Investigation and Formal Analysis: all authors; Writing – Original Draft: HYL; Writing – Review and Editing: all authors; Supervision: AKK and JAM; Funding Acquisition: HYL, JAM, AKK.

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CONFLICT OF INTEREST

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of the data; in the writing of the manuscript; or in the decision to publish the result.

DATA AVAILABILITY STATEMENT

Relevant genome data are available in GenBank at the accession numbers: GCA_003030465.1; GCA_012911005.1; JALLIK000000000; JALLIL000000000; JALLIM000000000; LARU01000005.1; SSXV01000143.1; WTVG00000000.2; WTVH00000000.2

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SUPPORTING INFORMATION

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