IncP-1β plasmids of *Comamonas* sp. and *Delftia* sp. strains isolated from a wastewater treatment plant mediate resistance to and decolorization of the triphenylmethane dye crystal violet

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The application of toxic triphenylmethane dyes such as crystal violet (CV) in various industrial processes leads to large amounts of dye-contaminated sludges that need to be detoxified. Specific bacteria residing in wastewater treatment plants (WWTPs) are able to degrade triphenylmethane dyes. The objective of this work was to gain insights into the genetic background of bacterial strains capable of CV degradation. Three bacterial strains isolated from a municipal WWTP harboured IncP-1β plasmids mediating resistance to and decolorization of CV. These isolates were assigned to the genera *Comamonas* and *Delftia*. The CV-resistance plasmid pKV29 from *Delftia* sp. KV29 was completely sequenced. In addition, nucleotide sequences of the accessory regions involved in conferring CV resistance were determined for plasmids pKV11 and pKV36 from the other two isolates. Plasmid pKV29 contains typical IncP-1β backbone modules that are highly similar to those of previously sequenced IncP-1β plasmids that confer antibiotic resistance, degradative capabilities or mercury resistance. The accessory regions located between the conjugative transfer (*tra*) and mating pair formation modules (*trb*) of all three plasmids analysed share common modules and include a triphenylmethane reductase gene, *tmr*, that is responsible for decolorization of CV. Moreover, these accessory regions encode other enzymes that are dispensable for CV degradation and hence are involved in so-far-unknown metabolic pathways. Analysis of plasmid-mediated degradation of CV in *Escherichia coli* by ultra-high-performance liquid chromatography-electrospray ionization-quadrupole-time-of-flight MS revealed that leuco crystal violet was the first degradation product. Michler’s ketone and 4-dimethylaminobenzaldehyde appeared as secondary degradation metabolites. Enzymes encoded in the *E. coli* chromosome seem to be responsible for cleavage of leuco crystal violet. Plasmid-mediated degradation of triphenylmethane dyes such as CV is an option for the biotechnological treatment of sludges contaminated with these dyes.
INTRODUCTION

The triphenylmethane dye crystal violet (CV) is used in several industrial processes and for different applications (Docampo & Moreno, 1990), e.g. in the textile industry to stain wool, cotton, silk and other raw materials, in paper printing (Azmi et al., 1998), as a reagent for bacterial classification (Kwasniewska, 1985), as an antifungal agent (Docampo & Moreno, 1990) and in cosmetic products (Jang et al., 2005). CV is harmful in different ways to humans and animals (Docampo & Moreno, 1990). In solution it is a cation able to interact with nucleophilic compounds such as negatively charged DNA (Littlefield et al., 1985). Binding of CV to DNA can lead to chromosomal damage and reduced expression of genes (Au et al., 1978), as well as increased growth of tumour cells in mice (Littlefield et al., 1985) and some fish (Chen et al., 2007). Moreover, CV can even cause blindness and kidney damage in humans (Mittal et al., 2010). The extensive application of CV and other triphenylmethane dyes, especially in the textile industry, leads to large amounts of contaminated wastewater, which need to be cleaned and detoxified before disposal to avoid environmental contamination, and animal and human health problems (Chen et al., 2008).

Several chemical and physical methods have been developed to degrade toxic compounds such as CV and other triphenylmethane dyes, but these processes are expensive and lead to large amounts of sludge (Azmi et al., 1998). Decolorization of triphenylmethane dyes and in particular CV by certain bacteria such as Nocardia corallina (Yatome et al., 1993), Mycobacterium avium (Jones & Falkinham, 2003), Aeromonas hydrophila (Ren et al., 2006) and Kurrthia sp. (Sani & Banerjee, 1999), as well as some fungi such as the white rot fungus Phanerochaete chrysosporium (Bumpus & Brock, 1988), represents an alternative biological solution. In the case of N. corallina (Yatome et al., 1993) and Bacillus subtilis (Yatome et al., 1991), Michler's ketone (MK) was detected as a CV degradation product. Analysis of CV degradation products released by Shewanella sp. also revealed MK and four other products (Chen et al., 2008). The enzyme triphenylmethane reductase, encoded by the tmr gene in Citrobacter sp. strain KCTC 18061P, was found to be responsible for decolorization of triphenylmethane dyes such as CV (Jang et al., 2005). A triphenylmethane reductase gene is also encoded in an accessory region of the IncP-1 plasmid pGNB1, which was isolated from a bacterium from a wastewater treatment plant (WWTP) (Schluter et al., 2007). This plasmid was found to confer resistance to and decolorization of CV.

To further analyse plasmid-mediated decolorization of triphenylmethane dyes and to elucidate corresponding degradation pathways, new CV-resistance plasmids were isolated from activated sludge bacteria from a WWTP. Sequencing of these plasmids revealed their genetic structure and putative genes that might function in degradative pathways. Metabolites arising in the course of plasmid-mediated CV degradation were further analysed by means of ultra-high-performance liquid chromatography-electrospray ionization-quadrupole-time-of-flight MS (UHPLC-ESI-Q-TOF-MS).

METHODS

Isolation of bacteria that are non-susceptible to CV from an activated sludge bacterial community. Activated sludge samples were taken from an activated sludge basin of the WWTP Bielefeld-Heepen (Germany) in March 2009. Activated sludge bacteria were cultivated on Luria broth (LB) agar medium containing CV (10 μg ml⁻¹) for 40 h at 25 °C. Growing bacteria were screened for clones able to decolorize CV (Schluter et al., 2007). Positive clones appear white and are surrounded by a colourless halo, indicating degradation of CV. In contrast, bacterial clones that are non-susceptible to CV feature an intensely violet colour.

Total plasmid DNA was isolated from positive clones by means of the NucleoBond PC 100 kit on AX 100 columns according to the protocol supplied by the manufacturer (Macherey-Nagel). Subsequently, plasmid DNA preparations were used to transform CaCl₂, competent Escherichia coli KAM3 (Morita et al., 1998) and DH5α cells. Transformants were selected on LB medium supplemented with CV (10 μg ml⁻¹). E. coli DH5α strains (Grant et al., 1990) containing plasmid pKV11, pKV29, pKV36, pGNB1 or pIK405 were grown in LB medium supplemented with CV (10 μg ml⁻¹) at 37 °C. For decolorization assays these strains were grown in 10 ml M9 minimal medium containing CV (10 μg ml⁻¹) for 24 h at 37 °C.

Standard DNA techniques. The plasmid content of candidate isolates and E. coli strains was analysed in Eckhardt gels as described elsewhere (Hynes et al., 1985). Plasmid DNA was isolated from E. coli strains by means of the NucleoBond PC 100 kit (Macherey-Nagel), whereas recombinant plasmids containing subcloned restriction fragments were isolated by using the QIAprep Spin Miniprep kit (Qiagen). Restriction enzyme digestion, agarose gel electrophoresis, DNA cloning and transformation of E. coli were carried out according to Sambrook et al. (1989).

Amplification of 16S rRNA gene fragments and part of the triphenylmethane reductase gene tmr. Crude total DNAs from activated sludge bacterial isolates were prepared as described previously (Schluter et al., 2007). 16S rDNA fragments were amplified from crude total DNA preparations by using the primers 1385r and 27f (Schlu¨ter et al., 2007). Taxonomic classification of 16S rDNA sequences was done by means of the RDP-Classifier (Wang et al., 2007) and by comparison with the NCBI nucleotide sequence database using the Blastn algorithm. The primer pair tmr-L1 (5’-CCCCATCATGAAACAACTGTA-3’) and tmr-R1 (5’-TCTTTTTTTTTTTTTTTTTTTTGGGATTTTTCCG-3’) was used to generate an amplicon containing only the complete pKV29 tmr gene encoding the enzyme triphenylmethane reductase. PCRs were done under standard conditions using the BIOTAQ DNA polymerase (Bioline). Amplicons were verified by sequencing. The amplicon containing the tmr gene from plasmid pKV29 was cloned into the T-cloning vector PGE-M-T Easy (Promega). The corresponding construct was named pIK405.

Complete sequencing of the CV-resistance plasmid pKV29. Plasmid DNA for sequence determination was isolated from E. coli DH5α (pKV29) using the Plasmid Midi kit (Qiagen) as low-copy-number plasmids. The DNA sequence of pKV29 was determined by pyrosequencing. The shotgun sequencing protocol using the Roche/454 platform has been described elsewhere (Margulies et al., 2005). The plasmid was sequenced together with...
29 other multiplex identifier (MID)-tagged libraries in a pool on a GS FLX platform with Titanium sequencing chemistry (Roche/454 Life Sciences). The number of reads obtained for plasmid pKV29 was 11,522 with an average read length of ~320 bp. The generated amount of sequence information accounted for a 60-fold coverage of the plasmid genome. Sequence reads were assembled using the Newbler assembly software (Roche/454 Life Sciences). Assembly of pKV29 reads resulted in two contigs. To complete the pKV29 sequence, a PCR-based strategy was applied, which involved PCR amplification of fragments covering gaps between contigs and sequencing of these amplicons.

**Annotation of plasmid sequences.** Annotation of plasmid sequences was done within the Annotation Platform GenDB 2.0 (Meyer et al., 2003) essentially as described previously (Eikmeyer et al., 2012; Heinl et al., 2012; Szczepanowski et al., 2011). Intergenic regions were analysed manually for missed coding sequences by applying BLAST programs (Altschul et al., 1997). Automatic annotations were curated manually if necessary. Plasmid DNA and amino acid sequences were compared with related plasmids deposited in the NCBI database by applying the tools BLASTN, BLASTP and BLASTX (Altschul et al., 1997).

**Restriction fragment library construction, sequencing and sequence analysis of the pKV11 and pKV36 CV-resistance gene regions.** Purified pKV11 and pKV36 plasmid DNA was restricted with the restriction endonucleases BamHI, EcoRI, PstI, SalI, SphI and NotI. Restriction fragments representing the CV-resistance gene region were separately subcloned into the sequencing vectors pUC18 (Yanisch-Perron et al., 1985), pBluescript-II-KS (Stratagene) and pZErO-2 (Invitrogen). Sequencing of recombinant plasmids was facilitated using the Consed/Autofinish software tool (Gordon et al., 1998, 2001). For annotation of the finished sequence, the annotation tool GenDB was applied (Meyer et al., 2003).

**Comparative analysis of accessory plasmid regions.** To determine homologous regions of the plasmids pKV11, pGNB1, pKV29 and pKV36, pairwise BLASTN analyses were performed. The sequences of accessory regions were extracted from plasmid sequences between the inverted repeat of the insertion sequence IS1071 and the stop codon of the second ORF located downstream of the triphenylmethane reductase gene *tmr*.

**Spectrophotometric measurement of plasmid-mediated decolorization of CV.** To follow decolorization of CV by *E. coli* DH5α (pIK405, *tmr*), 6 ml of overnight cultures (OD₆₀₀ 0.96) were separately transferred into 100 ml glucose M9 minimal medium (supplemented with thiamine) containing CV (5 µg ml⁻¹) and were shaken (150 r.p.m.) at 37 °C. Aliquots (1 ml) of the cultures were taken every 2 h beginning at time point zero (*t* = 0) until decolorization of CV had been completed (*t* = 10). Sampled aliquots were centrifuged and the absorbance of cell-free supernatants was measured from 200 to 700 nm (Ultrascan 3000 pro UV/visible spectrophotometer, GE Healthcare). A plasmid-free *E. coli* DH5α culture in M9 medium without CV was taken as control; M9 medium containing CV but no bacteria was taken as reference. For additional decolorization assays, the bacterial strains *Comamonas* sp. KV11, *Delftia* sp. KV29, *Comamonas* sp. KV36 and *E. coli* DH5α (pKV29) were cultivated in triplicate experiments in LB medium at room temperature. Samples were taken every 1.5 h and the absorbance of the supernatant was measured at 590 nm using LB medium without CV as a reference. CV concentrations were calculated from a calibration curve with known CV concentrations. CV degradation rates were calculated for each period between samplings.

**Extraction of CV degradation products by solid-phase extraction (SPE).** Aliquots (3 ml, OD₂₆₅ 1.1) of decolorized overnight cultures were transferred into 100 ml M9 minimal medium containing CV (80 µg ml⁻¹) and shaken (150 r.p.m.) at 37 °C until complete decolorization was achieved (~10 h). Cultures were centrifuged (4 °C, 8000 r.p.m.) and supernatants were filtered using 0.2 µm pore-size Whatman filters. CV degradation products were extracted by SPE. Supernatants were applied onto columns (Discovery DSC-C18, 1 ml, 100 mg, Supelco). After washing with water, the compounds were eluted with 1 ml acetonitrile (containing 0.1% formic acid) and stored in the dark at 4 °C.

**Analysis of CV degradation products by UHPLC-ESI-Q-TOF-MS.** Liquid chromatography (LC) separations of aliquots (3 µl) of the extracted degradation products were performed using an ACQUITY UHPLC system (Waters) combined with an ACQUITY UHPLC BEH C18, 1.7 µm column (2.1 x 50 mm, Waters). Eluent solvents were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The following gradient was applied at a flow rate of 0.35 ml min⁻¹: 0 to 0.5 min isocratic at 95% A; 0.5 to 6.5 min linear gradient from 95 to 5% A; 6.5 to 7.0 min isocratic at 95% A; 7.0 to 7.5 min linear gradient from 5 to 95% A. The total runtime was 10 min.

The UHPLC was hyphenated with an Apollo II ESI source operated in positive ion mode to a micrOTOF-QII ESI-Q-TOF mass spectrometer (Bruker Daltonics). Mass spectrometer source settings were as follows: dry gas temperature 200 °C, dry gas flow 8 l min⁻¹, nebulizer gas pressure 2 bar, capillary voltage 4500 V, end plate offset −500 V. Mass spectra were detected from 75 to 1000 m/z at 1 Hz acquisition speed.

The data were evaluated using the DataAnalysis 4.0 software (Bruker Daltonics). Target compounds were confirmed by comparison with purified standards and by calculating the sum formulae using the SmartFormula module in DataAnalysis 4.0, which takes into account accurate mass and isotopic pattern information.

**RESULTS AND DISCUSSION**

**Isolation of bacterial strains able to decolorize CV from an activated sludge community of a WWTP**

To isolate bacteria that were able to decolorize the triphenylmethane dye CV, aliquots of diluted activated sludge samples from a municipal WWTP were cultivated on LB agar medium containing CV to a final concentration of 10 µg ml⁻¹. Approximately 2.5% of activated sludge bacteria that were able to grow on LB medium were not susceptible to the CV concentration applied. Moreover, about 1.4% of those bacteria that could grow on LB medium supplemented with CV decolorized the dye. Decolorizing colonies were white, in contrast to dark violet colonies, which were not susceptible to CV but were unable to decolorize it. Thirty-six colonies that showed reduced susceptibility to CV and the decolorization phenotype were chosen for further analysis. Since in the first instance we were interested in plasmid-encoded decolorization of CV, the obtained isolates were initially tested for their plasmid
content by applying the Eckhardt gel lysis method. Eighteen out of 36 isolates appeared to contain plasmids. Plasmid-free isolates were discarded. To exclude putative pathogenic bacteria from further analysis, partial 16S rRNA gene sequences were determined for each isolate. Based on the analysis of these sequences, seven isolates were potentially pathogenic bacteria (Acinetobacter, Aeromonas, Klebsiella and Stenotrophomonas) and were therefore excluded from further analysis. The plasmids of five other isolates appeared to be unstable, so that analysis of these isolates was not feasible. The remaining six plasmid-containing isolates were named KV10, KV11, KV16, KV17, KV29 and KV36. The isolates KV11, KV29 and KV36 contained plasmids of about 60–70 kb in size, whereas the isolates KV10, KV16 and KV17 harboured large plasmids that had sizes of at least 150 kb (data not shown). At this stage it was not clear whether the CV decolorization phenotype of the isolates was plasmid or chromosomally encoded.

**Bacterial isolates able to decolorize CV are members of the family Comamonadaceae**

To taxonomically classify the activated sludge isolates that were able to decolorize CV, their 16S rRNA genes were partially sequenced. All isolates were allocated to the class β-Proteobacteria, the order Burkholderiales and the family Comamonadaceae (Table 1). The isolates KV11 and KV36 belonged to the genus Comamonas, whereas the other four isolates were affiliated to the genus Delftia. Many Delftia and Comamonas strains are able to degrade xenobiotics and other man-made compounds. Moreover, members of the genera Delftia and Comamonas are known to harbour plasmids involved in degradation of different recalcitrant substances.

**Isolates KV11, KV29 and KV36 contain CV-resistance plasmids**

To analyse whether the plasmids of the strains KV10, KV11, KV16, KV17, KV29 and KV36 were responsible for the observed CV-resistance and decolorization phenotype, plasmid DNA was isolated from each strain and subsequently used to transform E. coli KAM3 and E. coli DH5α. E. coli KAM3 carries a deletion in the multidrug efflux system genes acrAB and therefore is markedly sensitive to triphenylmethane dyes (Morita et al., 1998). E. coli transformants were selected on LB medium containing 10 μg CV ml⁻¹. Transformants were obtained for the plasmids pKV11, pKV29 and pKV36 of the isolates KV11, KV29 and KV36, respectively. Accordingly, these three plasmids conferred CV resistance to E. coli, suggesting that they carry a corresponding resistance gene. Moreover, E. coli derivatives carrying the plasmids pKV11, pKV29 and pKV36 were able to decolorize CV, which was apparent as a colourless halo surrounded growing colonies on agar plates containing CV (10 μg ml⁻¹). The plasmids of the isolates KV10, KV16 and KV17 could not be transformed into E. coli. Either the plasmids were too large to be efficiently transformed or replication of these plasmids was not compatible with the E. coli background. It is also possible that the CV-resistance phenotype of strains KV10, KV16 and KV17 is encoded on the chromosome and not on the plasmids. Since the involvement of plasmids pKV10, pKV16 and pKV17 in CV decolorization was unclear, further analyses focused on the plasmids pKV11, pKV29 and pKV36.

**IncP-1β plasmids pKV11, pKV29 and pKV36 are related to the reference plasmid pGNB1**

The CV-resistance reference plasmid pGNB1 (Schütler et al., 2007) belongs to the IncP-1β incompatibility group.

### Table 1. Taxonomic affiliation of activated sludge isolates with the ability to decolorize CV

<table>
<thead>
<tr>
<th>Isolate</th>
<th>RDP-Classifier assignment* †</th>
<th>Best BLASTN hit</th>
<th>Identity</th>
<th>Origin of reference strain</th>
<th>GenBank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>KV10</td>
<td>Delftia (100 %)</td>
<td>Delftia sp. XY6</td>
<td>1248/1249</td>
<td>Sediment of wastewater treatment in a pesticide plant, Beijing, China</td>
<td>EU707799</td>
</tr>
<tr>
<td>KV11</td>
<td>Comamonas (100 %)</td>
<td>Comamonas sp. H4-3</td>
<td>1244/1245</td>
<td>Community using poly(3-hydroxybutyrate-co-3-hydroxyvalerate) in solid-phase denitrification processes</td>
<td>AB277848</td>
</tr>
<tr>
<td>KV16</td>
<td>Delftia (100 %)</td>
<td>Delftia sp. JDC-3</td>
<td>1249/1249</td>
<td>Sediment of wastewater treatment in a pesticide plant, Beijing, China</td>
<td>FJ378038</td>
</tr>
<tr>
<td>KV17</td>
<td>Delftia (100 %)</td>
<td>Delftia sp. XY6</td>
<td>1248/1249</td>
<td>–</td>
<td>EU707799</td>
</tr>
<tr>
<td>KV29</td>
<td>Delftia (100 %)</td>
<td>Bacterium SE4</td>
<td>1248/1249</td>
<td>Gastrointestinal tract of Epinephelus coioides</td>
<td>EU520344</td>
</tr>
<tr>
<td>KV36</td>
<td>Comamonas (100 %)</td>
<td>Comamonas sp. H4-3</td>
<td>1244/1245</td>
<td>Community using poly(3-hydroxybutyrate-co-3-hydroxyvalerate) in solid-phase denitrification processes</td>
<td>AB277848</td>
</tr>
</tbody>
</table>

*All isolates were classified as belonging to the class β-Proteobacteria, the order Burkholderiales and the family Comamonadaceae by means of the RDP-Classifier.

†All RDP-Classifier results got the confidence value 100 %.
Therefore, we tested whether the plasmids pKV11, pKV29 and pKV36 also belong to this group. IncP-1-specific replication initiation trfA fragments were amplified by means of the trfA-specific primers trfA-1 and trfA-2 (Götz et al., 1996). Amplicons were obtained for all three plasmids, and sequencing of the trfA amplicons revealed that the fragments of all plasmids are almost identical to corresponding sequences of the CV-resistance reference plasmid pGNB1 and the Burkholderia ambifaria AMMD plasmid 1 (see Table 2). The latter plasmid also is an IncP-1 plasmid, but its precise function has not yet been determined (GenBank accession no. CP000443). Thus, the plasmids pKV11, pKV29 and pKV36 clearly belong to the IncP-1/β incompatibility group and their trfA nucleotide sequences cluster within the subgroup of plasmids represented by the catabolic plasmids pGNB1, pJP4 (Trefault et al., 2004), pUO1 (Sota et al., 2003) and pADP-1 (Martínez et al., 2001), the antibiotic-resistance plasmids pB8 (Schlüter et al., 2005) and pB10 (Schlüter et al., 2003), and the mercury-resistance plasmid pTP6 (Smalla et al., 2006).

As plasmids pKV11, pKV29 and pKV36 are of different sizes, it is assumed that they mainly differ in their accessory modules.

**Complete sequencing of the IncP-1/β plasmid pKV29 reveals that it contains an accessory CV-resistance gene region**

Plasmid pKV29 was sequenced at the DOE Joint Genome Institute (JGI) on the GS FLX platform. The finished plasmid genome appeared to be 61,667 bp in size, with an average GC content of 64.0%. It contains 69 predicted protein-coding sequences (see Fig. 1). Plasmid pKV29 consists of eight backbone modules involved in replication initiation (ssb–trfA), plasmid stabilization, maintenance and control (klu, klc, kle, incC–korB and kfr), conjugal plasmid transfer (tra) and mating pair formation (trb) (Fig. 1). These backbone modules are very similar or identical to corresponding modules of other IncP-1β plasmids such as the atrazine catabolic plasmid pAM1 (NC_004956), the B. ambifaria AMMD plasmid 1 (NC_008385) and different antibiotic-resistance plasmids (Table 3). Plasmid pKV29 harbours two accessory regions located downstream of the replication initiation gene trfA and between the tra and trb modules, respectively. The smaller accessory region downstream of the trfA gene contains seven genes and is flanked by inverted repeats related to those of transposon Tn5501 (inverted repeat Tn5501 in Fig. 1). The element inserted into pKV29 is homologous to the Tn5501-like element of the reference plasmid pGNB1 (Schlüter et al., 2007) and encodes a conserved barrel protein belonging to the Cupin 2 family and a putative addiction system (parE–orf1).

The second accessory region on pKV29 contains the tmr gene, encoding a putative triphenylmethane reductase (Tmr). The tmr gene region features a particularly low GC content (minimum ~34 %) compared with other parts of the plasmid, suggesting that this segment was acquired by the plasmid only recently. ORFs located up- and downstream of tmr encode hypothetical proteins that are identical to corresponding gene products of the reference plasmid pGNB1 (Fig. 2). The second pKV29 accessory region is flanked by a conserved module that consists of a copy of the insertion sequence IS1071, a truncated lpdA gene (dihydrolipoamide dehydrogenase) and a transposase gene (tnpA) of another insertion sequence element (ISGNB1-2). This module is also conserved in the reference plasmid pGNB1. The central part of the second pKV29 accessory region is composed of six coding sequences. Most of the corresponding gene products are hypothetical; only the product of the cobA gene was predictable: it encodes a putative uroporphyrinogen-III C-methyltransferase that is 84 % similar to a corresponding enzyme of the Hydra magnipapillata symbiont Curvibacter sp. (Comamonadaceae) (Chapman et al., 2010).

**pKV11 and pKV36 accessory gene regions encoding the triphenylmethane reductase gene tmr are related but not identical**

As deduced from partial sequencing of plasmid-specific genes, the backbone regions of the CV-resistance plasmids pKV11 and pKV36 are very similar in comparison with plasmid pKV29. Accordingly, only the accessory regions of these plasmids were sequenced. The pKV11 CV-resistance region was subcloned on an 11 kb BamHI and an overlapping 5.5 kb SphI restriction fragment. Both fragments mediated CV resistance and decolorization in E. coli DH5α. Therefore it was decided to determine the complete
nucleotide sequences of both restriction fragments. The SphI fragment contains the IncP-1-specific mating pair formation gene *trbN* followed by a truncated version of *trbO*. The arrangement of these genes is exactly the same as described for the reference plasmid pGNB1 (Schlüter et al., 2007). Downstream of the mating pair formation module genes on plasmid pKV11 there are four additional genes encoding a putative transcriptional regulator protein (Orf14), a conserved hypothetical protein (Orf13), a triphenylmethane reductase (Tmr) and a hypothetical protein (Orf11) (Fig. 2). The arrangement and sequences of these four genes are identical to those of the corresponding genes on plasmid pGNB1. The described module is flanked by an insertion of a truncated ISGNB1-2 element. Differences between plasmids

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**Fig. 1.** Genetic map of the IncP-1β CV-resistance plasmid pKV29. Coding regions are shown by arrows, indicating the direction of transcription. Coding sequences representing a specific plasmid module are differentiated by different colours. The origin of vegetative replication (oriV) is symbolized by a black circle, inverted repeats by black bars. GC content (orange/blue) and GC skew (red/green) are plotted on the inner circles. The first accessory plasmid region (red) located downstream of the replication module (*ssb–trbA*) is flanked by a truncated and a complete transposable element (*tnpA*) and contains five further genes similar to corresponding genes of the reference CV-resistance plasmid pGNB1 (Schlüter et al., 2007). The second accessory region of the plasmid (light green) located downstream of the *tra* module includes the triphenylmethane reductase gene *tmr*, which is involved in decolorization of triphenylmethane dyes.
pGNB1 and pKV11 were found beyond the ISGNB1-2 insertion. Plasmid pKV11 possesses three genes in this region which have no counterparts on pGNB1. These genes encode a protein of the glucose-methanol-choline (GMC) oxidoreductase family (Cavener, 1992), a conserved hypothetical protein and a putative post-segregational killing system. These genes may represent a second truncated module consisting of an incomplete ISgnb1 region are hypothetical. Moreover, plasmid pKV36 harbours a second truncated module consisting of an incomplete ISGNB1-2. Homologous recombination via two identical insertion sequence elements can potentially convert plasmid pKV36 into a pKV29-like or a pGNB1-like plasmid, respectively. However, it is not clear whether this conversion occurs in vivo.

In contrast, the accessory CV-resistance region of plasmid pKV36 (Fig. 2) is more related to the corresponding region of plasmid pKV29. The trb module and the IS1071-1lpdA gene region are conserved on pKV29. In addition, this plasmid also contains a cobA module composed of six genes that are also present on plasmid pKV29 (Fig. 2). However, plasmid pKV36 possesses accessory genes that are not present in any sequenced accessory region of the other plasmids. These pKV36 genes encode a short-chain dehydrogenase/reductase (sdr) and a hypothetical protein with highest similarity (79 %) to the Comamonas testosterone CNB-2 protein TctC (Ma et al., 2009). TctC is thought to be the extra-cytoplasmic receptor component of the tripartite tricarboxylate transporter family (pfam03401). The gene products of two further coding sequences in this region are hypothetical. Moreover, plasmid pKV36 harbours a second truncated module consisting of an incomplete IS1071 element, 'lpdA and a partial copy of ISGNB1-2. Homologous recombination via two identical insertion sequence elements can potentially convert plasmid pKV36 into a pKV29-like or a pGNB1-like plasmid, respectively. In summary, the accessory CV-resistance regions of plasmids pKV11, pGNB1, pKV29 and pKV36 are related to each other and are most probably derived from a common ancestor.

### The plasmid-encoded triphenylmethane reductase (Tmr) is responsible for decolorization of CV

The triphenylmethane reductase (Tmr) encoded on each of the pKV plasmids was thought to be the key enzyme for decolorization of CV. To analyse whether other genes identified on the pKV plasmids are involved in degradation of CV, decolorization assays were carried out. As a control, plasmid pIK405, containing a cloned copy of the complete tmr gene originating from plasmid pKV29, was included in the assays. E. coli DH5α strains harbouring plasmids...
pGNB1, pKV11, pKV29 and pKV36 were cultivated in M9 minimal medium supplemented with CV to a final concentration of 10 μg ml⁻¹. Samples (1 ml) were taken every 2 h and centrifuged, and the supernatant was measured by spectrophotometry at 200–700 nm. CV features characteristic absorption peaks at 212, 248, 303 and 590 nm (Fig. 3a). Plasmid-containing *E. coli* DH5α strains were able to decolorize the medium within 10 h (Fig. 3b).

The decolorization capacity was comparable for all *E. coli* derivatives tested, irrespective of the plasmid harboured by the cells. Also, plasmid plK405, which contains the *tmr* gene alone, conferred decolorization ability upon *E. coli* DH5α. This observation indicates that the triphenylmethane reductase gene *tmr* is necessary and sufficient for decolorization of CV in *E. coli*. The plasmid-free *E. coli* DH5α parental strain was unable to grow in the medium supplemented with CV and did not decolorize the dye. It appeared that none of the accessory genes identified on plasmids pKV11, pKV29, pKV36 and pGNB1 affected the decolorization efficiency of the corresponding *E. coli* DH5α derivatives. Accordingly, the triphenylmethane reductase (Tmr) is the key enzyme for decolorization of CV or at least catalyses the first step in this pathway. These observations are in line with earlier results obtained by Jang *et al.* (2005). These authors showed that a purified triphenylmethane reductase from a *Citrobacter* sp. strain was able to catalyse the NADH-dependent reduction of triphenylmethane dyes.

To compare the decolorization rate of *E. coli* DH5α (pKV29) with those of the original isolates *Comamonas* sp. KV11, *Delftia* sp. KV29 and *Comamonas* sp. KV39, decolorization assays were also done for these strains. All isolates were able to decolorize the medium (LB) containing CV (10 μg ml⁻¹) within 6 h. The maximum degradation rates varied between 0.957 and 1.258 mg l⁻¹ h⁻¹ (see Table 4), indicating that
Identification of CV degradation metabolites

Metabolites of the CV degradation process were analysed by UHPLC-ESI-Q-TOF-MS. Leuco crystal violet (leuco-CV), a reduced and colourless form of CV, was previously identified as the first product of CV degradation. The enzyme triphenylmethane reductase (Tmr) catalyses the conversion of CV to leuco-CV (Jang et al., 2005). Further possible degradation metabolites of CV are MK as well as 4-dimethylaminobenzaldehyde (Yatome et al., 1992).

Accordingly, these compounds were used as reference substances in liquid chromatography-MS (LC-MS) measurements (Fig. 4). Supernatants of decolorization assays with E. coli DH5α derivatives carrying plasmids pGNB1, pKV11, pKV29, pKV36 or pIK405 were analysed by UHPLC-ESI-Q-TOF-MS to elucidate the plasmid-encoded CV degradation pathway. The CV degradation metabolite profiles are shown as base peak chromatograms (BPCs) and as extracted ion chromatograms (EICs), also including extracted masses for reference substances. The EICs of all plasmid-mediated degradation reactions revealed the same composition and quantity of degradation products in the supernatants extracted. An example of a metabolite profile is shown for E. coli DH5α carrying plasmid pIK405, which only encodes the triphenylmethane reductase gene tmr (Fig. 4). Leuco-CV, MK and 4-dimethylaminobenzaldehyde were found in the culture supernatants after degradation of CV. The CV peak itself was no longer detectable after 10 h of incubation, indicating quantitative degradation of CV. The doubly protonated form of leuco-CV had a low peak intensity and was found to elute at 2.3 min (Fig. 4d, 1). Peak 2 had a similar accurate mass and the same elemental composition (C₉H₁₂N₁O₁) as peak 3. The latter had a retention time of 3.17 min and corresponds to p-dimethylaminobenzaldehyde, as demonstrated in comparison with the purified standard (Fig. 4h). The MK standard eluted at 4.55 min (Fig. 4g). Dimethylaminobenzaldehyde and MK peak intensities were lower for the E. coli (pIK405) supernatant (Fig. 4d) as compared with the leuco-CV peak. This observation suggests that leuco-CV was degraded into further compounds. Due to its relatively high peak intensity, it might be suggested that MK is one of the end-products of this reaction cascade in E. coli, whereas 4-dimethylaminobenzaldehyde may be further degraded into compounds that so far cannot be identified (Fig. 5). Since the metabolite profile of the E. coli

Table 4. CV degradation rates of the original isolates Comamonas sp. KV11, Delftia sp. KV29 and Comamonas sp. KV36 in comparison with E. coli DH5α (pKV29)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Maximum CV degradation rate (mg l⁻¹ h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Comamonas sp. KV11</td>
<td>1.258</td>
</tr>
<tr>
<td>Delftia sp. KV29</td>
<td>0.957</td>
</tr>
<tr>
<td>Comamonas sp. KV36</td>
<td>0.974</td>
</tr>
<tr>
<td>E. coli DH5α (pKV29)</td>
<td>1.063</td>
</tr>
</tbody>
</table>
DH5α derivative harbouring plasmid pIK405, which only contains the triphenylmethane reductase gene *tmr*, is indistinguishable from those of the other strains, it has to be concluded that enzymes encoded in the *E. coli* chromosome are involved in the leuco-CV degradation cascade. The NADH-dependent reduction of CV to leuco-CV by the enzyme triphenylmethane reductase (Tmr) has been demonstrated unambiguously *in vitro* (Jang et al., 2005). Currently, there are no indications that triphenylmethane reductase possesses further enzymic activity except for reduction of triphenylmethane dyes. However, *E. coli* is known for its capacity to degrade certain aromatic compounds (Díaz et al., 2001), although candidate enzymes involved in leuco-CV decomposition have not been identified so far.

**Diversity and evolution of WWTP plasmids conferring degradation of CV**

Specific autochthonous bacteria residing in the activated sludge community of a municipal WWTP are able to decolorize and thereby at least to some extent detoxify the triphenylmethane dye CV. For the bacterial isolates described in this study, resistance to and decolorization of the dye are associated with the presence of the plasmid-born triphenylmethane reductase gene *tmr*. This gene has probably been captured by the plasmids only recently, since its G+C content differs considerably from the rest of the plasmid. Interestingly, *tmr* is the only gene on the plasmids analysed that has a proven function in triphenylmethane
dye degradation. Obviously, the corresponding gene product triphenylmethane reductase renders the triphenylmethane molecule accessible for further degradation by enzymes that are not encoded on the plasmids. Most probably, chromosomally encoded enzymes functioning in aromatic compound degradation accomplish the further decomposition of the leuco form of CV in E. coli DH5α carrying the CV-resistance plasmids pKV11, pKV29 or pKV36. Currently, the original function of these plasmids remains unknown. In addition to the tmr gene, all the plasmids possess further accessory genes. However, although for some of these genes functional predictions were obtained, it was not possible to deduce a general functional context or metabolic pathway in which corresponding gene products were involved. It cannot be excluded that enzymatic functions encoded on the plasmids complement chromosomally encoded metabolic pathways of the host bacterium. Contrary to this assumption is the fact that IncP-1 plasmids usually possess a very broad host range. For the IncP-1/par CV-resistance plasmid pGNB1 it has been shown that it is self-transmissible and can be conjugated to z- and γ-proteobacteria, and therefore is a broad-host-range plasmid (Schlüter et al., 2007). It is unlikely that plasmid-encoded traits are compatible with different chromosomal backgrounds. However, the CV-resistance and decolorization phenotype mediated by plasmid pGNB1 is also expressed in the z-proteobacterium Sinorhizobium meliloti (Schlüter et al., 2007), indicating that the promoter of the triphenylmethane reductase gene is functional in different host bacteria. The activity of the triphenylmethane reductase seems to be very specific, since it accepts malachite green, CV and basic fuchsin as substrates, but it does not catalyse the reduction of brilliant green, bromophenol blue and azo dyes (Jang et al., 2005). However, it is conceivable that triphenylmethane reductase is also able to reduce naturally occurring compounds featuring a chemical structure that is similar to that of triphenylmethane dyes. In summary, reduction of triphenylmethane dyes seems to be a secondary function of the plasmids analysed in this study. The primary function of these plasmids remains unknown so far.

Comparative analysis of the accessory regions of the plasmids pKV11, pKV29, pKV36 and pGNB1 helped in understanding the evolution of these plasmids. Obviously, insertion sequences played a role in the acquisition of new plasmid modules, since recognizable units are flanked by either ISGNB1-2 or IS1071. Recombination events via identical insertion sequence elements can lead to deletion or inversion of certain accessory modules, depending on the orientation of flanking insertion sequences. Since all transposase genes (tnpA) of the insertion sequence elements located on the plasmids analysed here are transcribed in the same direction, homologous recombination via identical insertion sequence copies will cause deletion of modules. Accordingly, it is assumed that the plasmids pKV11, pKV29, pKV36 and pGNB1 originate from a common ancestor which step by step acquired additional modules, to develop the composition that has been achieved in plasmid pKV36, for example. Thus, insertion sequence elements in accessory plasmid modules ensure flexibility and facilitate rapid adaptive responses to changing environmental conditions. However, the driving force(s) in the evolution of the pKV plasmids is not yet clear. In any case, acquisition of the triphenylmethane reductase gene (tmr) seems to be an important evolutionary event, since all plasmids analysed in this study contain this gene, suggesting that it represents an adaptive advantage.

In conclusion, WWTP bacteria harbour diverse mobile genetic elements specifying degradation capabilities that might be exploitable in biotechnological processes designed for the bioremediation of dye-contaminated industrial wastes.

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