Genomics of IncP-1 antibiotic resistance plasmids isolated from wastewater treatment plants provides evidence for a widely accessible drug resistance gene pool

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Abstract
The dramatic spread of antibiotic resistance is a crisis in the treatment of infectious diseases that affect humans. Several studies suggest that wastewater treatment plants (WWTP) are reservoirs for diverse mobile antibiotic resistance elements. This review summarizes findings derived from genomic analysis of IncP-1 resistance plasmids isolated from WWTP bacteria. Plasmids that belong to the IncP-1 group are self-transmissible, and transfer to and replicate in a wide range of hosts. Their backbone functions are described with respect to their impact on vegetative replication, stable maintenance and inheritance, mobility and plasmid control. Accessory genetic modules, mainly representing mobile genetic elements, are integrated in-between functional plasmid backbone modules. These elements carry determinants conferring resistance to nearly all clinically relevant antimicrobial drug classes, to heavy metals, and to quaternary ammonium compounds used as disinfectants. All plasmids analysed here contain integrons that potentially facilitate integration, exchange and dissemination of resistance gene cassettes. Comparative genomics of accessory modules located on plasmids from WWTP and corresponding modules previously identified in other bacterial genomes revealed that animal, human and plant pathogens and other bacteria isolated from different habitats share a common pool of resistance determinants.

Introduction: impact of wastewater treatment plants on the horizontal transfer of mobile genetic elements conferring resistance

Increasing resistance of human pathogens to antimicrobial agents threatens to reverse progress in the treatment of infectious diseases. In particular, hospital-acquired (nosocomial) infections caused by multiresistant bacterial pathogens are a serious problem in intensive care units (Goosens, 2005; Lim & Webb, 2005). Resistance to antibiotics was and still is selected for by overuse and misuse of these agents in human and veterinary medicine, as growth promoters in animal production and for the treatment of bacterial plant diseases (Bywater, 2004, 2005; Finch, 2004; Wassenaar, 2005). Bacteria have evolved different strategies to cope with severe effects caused by antimicrobial drugs (Davies, 1994; Rybak, 2004; Sheldon, 2005). Intrinsically resistant species for example render their cell walls impermeable for antibiotics or express efflux systems facilitating transport of these compounds out of the cell. Moreover, bacteria are able to horizontally acquire resistance via uptake of foreign DNA by means of conjugation, transduction or transformation (Davison, 1999; Thomas & Nielsen, 2005). In this context mobile genetic elements (MGE) such as plasmids, transposable elements or integron-specific gene cassettes play an important role (Toussaint & Merlin, 2002; Frost et al., 2005). These elements mainly encode enzymes for modification or inactivation of antibiotics, efflux systems, or enzymes catalysing target-site modifications (Mazel & Davies, 1999). In recent years multiple examples of genetic exchange between bacteria residing in different habitats have been reported, and it is generally believed that bacteria participate in common gene pools and circulate genetic information (Davies, 1994; Severo et al., 2002; Thomas & Nielsen, 2005).

Wastewater treatment plants (WWTP) are of particular significance as interfaces between different environmental compartments, such as hospitals and surface waters, and
therefore may facilitate gene spread between these habitats. Biofilm and flock formation, high bacterial densities and metabolic activities in the aeration and clarifiers allow – and may even promote – genetic exchange by conjugation in these treatment facilities (Mach & Grimes, 1982; Mancini et al., 1987). Over the past 20 years several studies have provided evidence that WWTP serve as important reservoirs for MGE encoding drug resistance (McPherson & Galt, 1986; Mancini et al., 1987; Galt, 1988; Fujita et al., 1993; Bauda et al., 1995; Dröge et al., 2000; Heuer et al., 2002; Smalla & Sobecky, 2002; Tennstedt et al., 2003, 2005; Szczepanowski et al., 2004a; Szczepanowski et al., 2005; Bönnemann et al., 2006). Among those MGE, several are broad-host-range (BHR) plasmids, i.e. plasmids that can transfer to and replicate in a broad range of phylogenetically distinct hosts. Even when BHR plasmids do not encode drug resistance, they can still mobilize nonself-transferable antibiotic resistance plasmids (Top et al., 1994; Boon et al., 2001). WWTP thus seem to facilitate recombination and dissemination of genes that encode adaptive traits such as antimicrobial resistance. Moreover, the presence of pollutants such as drugs, xenobiotics, surfactants, heavy metals, etc. in sewage (Kümmerer, 2001, 2003) selects for the maintenance of elements conferring degradation of or resistance to these compounds. In order to better understand the diversity, spread and persistence of mobile drug resistance plasmids in the environment, a closer look at the extant pool of BHR conjugative plasmids in WWTP seems appropriate and timely.

Plasmids of the incompatibility group IncP-1 (also referred to as IncP in the Escherichia coli classification) currently are the best-studied BHR plasmids, and thought to be the most promiscuous of all plasmids known to-date. BHR plasmids and IncP-1 plasmids in particular have drawn considerable attention because they can spread genetic information across taxonomic barriers and thus contribute to the rapid adaptation of various bacterial populations in natural and clinical environments (Thomas, 2000b). In addition to antibiotic resistance determinants they often carry mercury resistance genes and genes that encode degradation of chlorinated organic compounds. IncP plasmids are geographically widespread today, and found in different subgroups of the proteobacteria (Alpha, Beta, Gamma), including enteric species. Since they were not found among enteric bacteria isolated before the clinical use of antibiotics (Datta & Hughes, 1983), the intensive use of these antimicrobials may have resulted in rapid spread from other species, where they may have originally benefited through resistance to mercury and other toxic compounds (Smith & Thomas, 1989). They are not only able to transfer and replicate in a wide range of hosts, but they can also mobilize nonself-transferable BHR IncQ plasmids, as well as so-called 'shuttle plasmids' to an even broader range of hosts, such as Gram-positive bacteria (Mazodier et al., 1989), cyanobacteria (Kreps et al., 1990), or even Eukaryotes such as yeast (Heinemann & Sprague, 1989).

At least four subgroups have currently been defined within the IncP-1 plasmid group, and complete genome sequences of their representatives are now available. IncP-1α plasmids were originally isolated from the Burns Unit of the Birmingham Accident Hospital in 1969 from multiresistant Pseudomonas aeruginosa and Enterobacter aerogenes (formerly Klebsiella aerogenes) strains (Thomas, 1981; Thomas & Smith, 1987). A set of five plasmids, namely R18, R68, RK2, RP1 and RP4, which could not be differentiated by restriction profile analysis nor by heteroduplex experiments (Burkardt et al., 1979; Currier & Morgan, 1981) are now referred to as the core 'Birmingham' IncP-1α plasmids. The first step towards genomics of IncP-1α plasmids was the compilation and assembly of available nucleotide sequence data for the 'Birmingham' plasmids, which resulted in a continuous circular sequence for a prototype IncP-1α plasmid (Pansegraup et al., 1994). The prototype plasmid of the second IncP-1 subgroup, IncP-1β, was isolated from a trimethoprim resistant Ent. aerogenes derivative (Johanpura & Datta, 1974). The complete nucleotide sequence for this plasmid, designated R751, became available in 1998 (Thorsted et al., 1998) and demonstrated that the R751 backbone parts share a similar genetic organization with the IncP-1α prototype plasmids. Subsequently, many more antibiotic resistance IncP-1α and -β plasmids were isolated but until recently (2001) none of them had been subjected to complete genome sequence analysis. More recently, three additional subgroups have been defined (γ-, δ-, and possibly e), mostly based on the phylogenetic relatedness of the TrfA proteins (Vedler et al., 2004; Haines et al., 2006; Bahl et al., 2007). More plasmid genomic data and comparative analyses are needed to fully understand the evolutionary relationships between these subgroups.

Over the past years different conjugative multiresistance plasmids have been obtained from municipal WWTP by the so-called exogenous isolation method, which involved transfer of plasmids from indigenous activated sludge bacteria to genetically marked recipients strains (see also section before Conclusions) (Dröge et al., 2000; Heuer et al., 2002; van Overbeek et al., 2002; Tennstedt et al., 2003; Tennstedt et al., 2005; Bahl et al., 2007). For example, different gentamicin resistance plasmids isolated exogenously from sewage were classified as belonging to the IncP-1β subfamily (Heuer et al., 2002). To date, six self-transmissible, broad-host-range IncP-1 plasmids originating from activated sludge bacteria have been completely sequenced (see Table 1). These are the IncP-1β plasmids pB2/pB3 (Heuer et al., 2004), pB4 (Tauch et al., 2003), pB8 (Schlüter et al., 2005) and pB10 (Schlüter et al., 2003) and the IncP-1α plasmid pTB11 (Tennstedt et al., 2005). In addition, the
genetic load region of the IncP-1β macrolide resistance plasmid pRSB111 isolated from bacteria residing in the final effluents of a WWTP was analysed at the nucleotide sequence level (Szczechawski et al., 2007). Accessory modules on these plasmids mainly consist of mobile genetic elements that carry diverse determinants conferring resistance to different aminoglycosides, β-lactams, macrolides, sulphonamides, tetracycline, trimethoprim, disinfectants and mercury ions.

Here insights gained by comparative genomic analysis of completely sequenced IncP-1 plasmids isolated from sewage sludge bacteria and plasmids isolated from clinical and other environments are reviewed. Different types of resistance modules, their genetic organization and insertion into plasmid backbone structures are described. These analyses provide clear evidence of genetic exchange between human, animal and plant pathogens and environmental bacteria including bacteria residing in wastewater habitats.

**IncP-1 resistance plasmids show a modular composition and accessory genetic elements are integrated in-between plasmid backbone modules**

IncP-1 plasmids show a modular backbone structure with accessory genetic elements integrated into the backbone. Since complete nucleotide sequences are available today for 19 different IncP-1 plasmids, their gene content and plasmid architecture structure can be analysed in detail. All plasmids contain genes for replication initiation (Rep), conjugative DNA-transfer (Tra) and mating-pair-formation (Trb), stable plasmid inheritance and plasmid control (CtI) in similar arrangements (see Figs 1 and 2). IncP-1α plasmids possess several genes that are missing on IncP-1β plasmids. These are _kleCD_ of the _Kle_ locus (possible accessory genes for active partitioning) (Kornacki et al., 1993; Wilson et al., 1997), _klaABC_ of the _Kla_ locus (involved in plasmid maintenance and fertility inhibition of IncW plasmids) (Goncharoff et al., 1991; Walter et al., 1991), the regulatory genes _korF_ and _korG_ encoding putative histone-like proteins (Jagura-Burzyk et al., 1991), the DNA-transfer genes _traAB_ (no functions assigned), the postsegregational-killing genes _parDE_ (Roberts & Helinski, 1992) and the mutlimer-resolution genes _parCBA_ (Sia et al., 1995). In contrast, _upf30_ and _upf31.0_ located downstream of _trbP_ and encoding, respectively, a putative outer membrane protein and a site-specific methylase were only found on IncP-1β plasmids and are missing on members of the IncP-1α subgroup (Thorsted et al., 1998). In addition, plasmids pB10, pAB1 and pJP4 encode a RelB/RelE family system representing a toxin/antitoxin addiction system (see below) that is not present on IncP-1α plasmids.

Figure 2 shows that comparison of the three sequenced IncP-1α antibiotic resistance plasmids (RP4, pTB11, pBS228) and the 13 sequenced IncP-1β plasmids confirms the 20-year old assumption, based then on restriction fragment patterns, that their structure is modular, with mobile accessory genetic elements inserted in-between functional plasmid backbone modules: usually between the _Tra_ and _Trb_ regions and/or in the oriV- _trfA_ region (Smith & Thomas, 1987). The 13 IncP-1β plasmids consist of six drug resistance plasmids [R751, pB2/pB3 (identical except for

**Table 1.** Completely sequenced IncP-1 multiresistance plasmids from wastewater treatment plant bacteria

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Inc-group</th>
<th>Size (bp)</th>
<th>Mobility</th>
<th>Resistance genes</th>
<th>Integron (ln)</th>
<th>Additional accessory functions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTB11</td>
<td>IncP-1α</td>
<td>68 869</td>
<td>Self-transmissible</td>
<td><em>aphA, aadA1, aacA4, oxa2, tetA</em></td>
<td>Class 1</td>
<td>orfs of unknown function</td>
<td>Tennstedt et al. (2005)</td>
</tr>
<tr>
<td>pB2/pB3</td>
<td>IncP-1β</td>
<td>60 732/56 167</td>
<td>Self-transmissible</td>
<td>_aadA2, <em>bla_NPS</em> _1, _cmA1, sul1, _tetA(C), <em>qacEA1</em></td>
<td>Class 1, Remnant Tn402</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>pB4</td>
<td>IncP-1β</td>
<td>79 370</td>
<td>Self-transmissible</td>
<td><em>bla_NPS</em> _1, _strAB, _mexCD-oprJ, <em>chrBAC</em></td>
<td>Remnant Tn402</td>
<td>orfs of unknown function</td>
<td>–</td>
</tr>
<tr>
<td>pB8</td>
<td>IncP-1β</td>
<td>57 198</td>
<td>Self-transmissible</td>
<td>_aadA4, _oxa2, _sul1, _qacEA1, <em>qacF</em></td>
<td>Class 1</td>
<td>Accessory stabilization modules, orfs of unknown function</td>
<td>Schlüter et al. (2003)</td>
</tr>
<tr>
<td>pB10</td>
<td>IncP-1β</td>
<td>64 508</td>
<td>Self-transmissible</td>
<td>_oxa2, _sul1, _strAB, _tetA, _qacEA1, <em>mer</em></td>
<td>Class 1</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*Incompatibility group.

1. _aphA, aadA1, aadA2, aadA4, aacA4, strAB confer aminoglycoside resistance; _oxa2, _bla_NPS_ _1, _cmA1, sul1, _tetA(C), _qacEA1_ confer β-lactam resistance; _cmA1_ confers chloramphenicol resistance; _sul1_ confers sulphonamide resistance; _tetA and _tetA(C) confer tetracycline resistance; _qacEA1 and _qacF_ confer resistance to quaternary ammonium compounds, _mer_ confers mercury resistance, _chrBAC_ is a putative chromate resistance determinant, _mexCD-oprJ_ encodes a tripartite multidrug resistance (MDR) efflux system.

Fig. 1. Genetic maps of the IncP-1β antibiotic resistance plasmids pB3, pB4, pB8 and pB10 and the IncP-1α resistance plasmid pTB11 isolated from WWTP bacteria. Plasmid backbone modules for replication initiation (Rep–grey), mating-pair-formation (Trb–green), conjugative DNA-transfer (Tra–red) and plasmid control and stable maintenance and inheritance (Ctl–yellow) are indicated by coloured bars. Insertions of accessory genetic elements are shown as lilac regions. These elements are always inserted in-between functional backbone modules. Further information on the encoded gene products is given in the text. Plasmid maps were redrawn from: (Heuer et al., 2004) (pB3), (Tauch et al., 2003) (pB4), (Schlüter et al., 2005) (pB8), (Schlüter et al., 2003) (pB10) and (Tennstedt et al., 2005) (pTB11).
Fig. 2. Modular structure of completely sequenced IncP-1 plasmids. Plasmid backbone modules for replication initiation (grey), mating-pair-formation (green), conjugative DNA-transfer (red) and plasmid control and stable maintenance and inheritance (yellow) are indicated by coloured bars. Insertions of accessory genetic elements are shown as lilac bars above the plasmid backbone structure, where disconnected bars indicate elements larger in size than 10 kb. These elements almost always inserted in-between functional backbone modules. Some plasmids possess either a complete or a truncated partition gene parA between Trb and Tra. Only the IncP-1α plasmids harbour complete parDE and parCBA operons. References for the different IncP-1 plasmids are: RP4 (Pansegraue et al., 1994), pTB11 (Tennstedt et al., 2005), pBS228 (Haines et al., 2007) R751 (Thorsted et al., 1998), pB3 (Heuer et al., 2004), pB4 (Tauch et al., 2003), pB8 (Schlüter et al., 2005), pB10 (Schlüter et al., 2005), pTP6 (Smalla et al., 2006), pJP4 (Trefault et al., 2004), pADP-1 (Martinez et al., 2001), pUO1 (Sota et al., 2003), pA81 (Jencova et al., 2004), pA1 (Harada et al., 2006), pBP136 (Kamachi et al., 2006), pQKH54 (Haines et al., 2006), pEST4011 (Vedler et al., 2004), and pKJK5 (Bahl et al., 2007).

duplication of a tetracycline resistance fragment in pB2), pB4, pB8, pB10), one mercury resistance plasmid (pTP6), four degradative plasmids (pADP-1, pJP4, pUO1 and pA81), and two so-called ‘cryptic’ plasmids with unknown phenotypic traits (pA1 and pBP136). Interestingly, very similar plasmid architecture is now found in the recently sequenced IncP-1γ mercury resistance plasmid pQKH54, the IncP-1δ degradative plasmid pEST4011, and the resistance plasmid pKJK5, which seems to belong to a fifth IncP-1 subgroup (see Fig. 2 for references or accession numbers for all 19 plasmids). Thus none of the backbone modules in IncP-1 plasmids are interrupted by insertions of accessory genetic elements, as these are always found integrated in intergenic regions between backbone modules. One exception however is the recently sequenced IncP-1γ plasmid pBS228, which carries a third accessory mobile element (Tn1013) that disrupted trbE in the Trb region (Fig. 2). pB4 is the only plasmid known to carry a transposable element (Tn5393c) in the region between the traKLM and kfrABC operons. Interestingly, none of the plasmids analysed at the nucleotide sequence level so far carry an accessory element between ssb-trfA and trb. This is most probably due to the complex regulation of vegetative replication and conjugative transfer replication that operates at regulatory sequences associated with promoters of the aforementioned operons. The promoters, trfAp and trbAp, for the divergently
transcribed operons ssb-trfA and trb and operator motifs for the repressors KorA and KorB play an important role in this regulation. Insertions of mobile elements into these regulatory motifs could disturb coordinate control of plasmid replication and conjugative DNA-transfer. Similarly, insertion in or between other backbone genes may affect plasmid replication, stability, transferability, or host fitness, as recently demonstrated for some transposon insertions into pBP136 (Sota et al., 2007). This study showed that a combination of region-specific insertion of transposons and selection for functional plasmids can explain the conserved architecture of the currently known IncP-1 plasmid family.

It had been suggested before, based on restriction fragment analysis, that the oriV-trfA and Tra/Trb intergenic loci may have been contiguous in a common ancestor of IncP-1 plasmids (Smith & Thomas, 1987, 1989). These studies also suggested that the Tra/Trb intergenic region is still contiguous in plasmid pJP4, which encodes degradation of the herbicide 2,4-dichlorophenoxyacetic acid. Seventeen years later, the complete genome sequence of plasmid pJP4 indeed confirmed that it is devoid of any interrupting mobile elements between the Tra and Trb regions (Trefault et al., 2004). The first example of a contiguous oriV-trfA region was found in the completely sequenced IncP-1β plasmids pB2 and pB3. Even remnants or footprints of mobile genetic elements could not be detected in the pB2/pB3 oriV region (Heuer et al., 2004). Therefore it was suggested that pJP4 and pB2/pB3 represent ancestral forms of the IncP-1 Tra/Trb and oriV-trfA regions, respectively. Very recently, two IncP-1β plasmids that contain no inserted mobile genetic elements were discovered. They are plasmid pA1 from Sphingomonas sp. A1 (Harada et al., 2006) and plasmid pBP136 from the human pathogen Bordetella pertussis (Komachi et al., 2006). Plasmid pA1 encodes two non-IncP-1-specific genes between Tra and Trb, which encode MIpA, predicted to interact with a membrane-bound lytic transglycosylase (pfam06629), and a transcriptional regulator of the TetR/AcrR family (pfam00440, COG1309). These two genes are not present on any other IncP-1β plasmid. Plasmid pBP136 also carries two non-IncP-1-specific ORFs (orf1 and orf2), but in a different location, between oriV and klcA. The respective gene products show similarity with the hypothetical proteins Xf1596 and Xf1597 found in the plant pathogen Xylella fastidiosa. Since all other IncP-1β plasmids carry independent insertions of accessory genetic elements in the oriV-trfA region and/or between the Tra and Trb regions, and pBP136 does not, this plasmid may well represent an ancestral form of IncP-1β plasmids, from which some of the currently known plasmids evolved through capture of different elements encoding various selectable phenotypic traits (Komachi et al., 2006).

In summary, IncP-1 resistance plasmids consist of different backbone modules facilitating plasmid replication, stable maintenance and inheritance, plasmid control and mobility. These replicons are usually enlarged by incorporation of accessory modules that carry mostly catabolic genes, mercury or drug resistance determinants. So far, plasmids with combinations of mercury resistance and either drug resistance or degradative operons have been found, but no IncP-1 plasmid that carries both drug resistance and degradative traits has yet been described. Thus IncP-1 plasmids seem to serve as vehicles for the dissemination of accessory modules that confer beneficial traits to their hosts. This probably allows them to be maintained in a bacterial community in spite of low but positive segregational loss rates, and a small but often non-zero cost to their hosts in the absence of selection for these traits. The role and persistence of IncP-1 plasmids like pA1 and pBP136 that encode no obvious beneficial traits for their bacterial hosts, is currently not understood. Functional aspects of several plasmid backbone modules are described in some more detail below.

**IncP-1 plasmid backbone functions**

**Inheritance of resistance determinants is ensured by plasmid encoded stabilization modules**

Plasmids usually encode stabilization systems ensuring their stable maintenance and inheritance. In principle, three different stabilization mechanisms have been described so far: (1) active partitioning of plasmid copies into daughter cells during cell division (PAR), (2) postsegregational-killing of plasmid-free segregants (PSK) and (3) resolution of plasmid multimers (MRS), (Zielienkiewicz & Cegłowski, 2001; Adamczyk & Jagura-Bledzka, 2003).

Active partitioning involves two protein components, IncC and KorB, and a cis-acting site on the plasmid (Williams & Thomas, 1992; Bignell et al., 1999; Bignell & Thomas, 2001). KorB (termed ParB on other plasmids) is a DNA-binding protein and recognizes the cis-acting site. IncC (termed ParA on other plasmids) possesses all characteristic features of NTPases, interacts with KorB and energizes the partitioning process through NTP-hydrolysis. In addition, the products of the kfrABC operon play a role in stable inheritance and plasmid survival (Adamczyk et al., 2006). The DNA-binding protein KfrA contains an α-helical, coiled-coil tail and together with KfrB and KfrC may act as a specific plasmid nucleoid organizer (Adamczyk et al., 2006).

Genes for active partitioning systems were found on all IncP-1 plasmids (see Table 2). Gene products encoded in the klc/kle region of IncP-1 plasmids are thought to modulate efficiency of active partitioning (Wilson et al., 1997; Thorsted et al., 1998). IncP-1β plasmids can be differentiated with respect to their kle gene regions. Plasmid pBP136 has only
two of the *kle* genes (*kleAE*), plasmids pB4 and pB10 only possess *kleAEF*, whereas pUO1 and pB2/3 have *kleABEF*, and pB8, R751 and pADP-1 even have *kleABEFG*. The biological significance of Kle gene products for the stability of these different IncP-1 plasmids is not well understood.

Postsegregational-killing (PSK) systems function based on a toxin/antitoxin principle (Roberts & Helsinki, 1992; Roberts et al., 1993; Sia et al., 1995). One component of the system is a toxin, for example ParE of IncP-1α plasmids, which inactivates DNA-gyrase thus inhibiting DNA-replication (Jiang et al., 2002). The second component, namely ParD of IncP-1α plasmids, serves as antidote of ParE toxicity and protects the cell from cell death. Antidotes either block the toxin at the protein level by direct interaction with the toxic component, or inhibit toxin synthesis. Presence of PSK systems results in killing of plasmid-free daughter cells as they still contain the stable toxin but have not received a plasmid copy expressing the unstable antitoxin. This is thought to promote plasmid stability, as plasmid-free segregants can no longer compete with plasmid-containing cells in the absence of selection for plasmid-encoded traits. It has also recently been suggested that PSK systems may have evolved to mediate the exclusion of competing plasmids, and not at all as plasmid-stabilizing systems (Cooper & Heinemann, 2000, 2005). This hypothesis was based on the observation that the PSK system did not allow the fraction of plasmid-containing cells to increase in competition with segregants. While PSK may very well play an important role in competition between plasmids, these recent findings do not exclude its role in overall plasmid stability. The plasmid used in this study did not confer a significant fitness cost to its host, while there are now several examples of hosts where IncP-1 plasmids confer a small to very large cost (De Gelder et al., 2007). In those populations, killing segregants that would otherwise outcompete their plasmid-containing counterparts in the absence of selection would still benefit plasmid persistence. Genes that potentially encode PSK-systems are present on all plasmids analysed here with the exception of the IncP-1β plasmids pB2/pB3 and pB4 (see Table 2). However, the role of these genes in postsegregational killing has not been confirmed. On the IncP-1β plasmid pB10 the gene products of *din* and the downstream *orf* belong to the RelB/RelE family representing a toxin/antitoxin addiction system. In accordance with the putative presence of two stabilization systems on pB10, very stable maintenance and inheritance of this plasmid has been observed in *Escherichia coli* (De Gelder et al., 2004) and in various strains belonging to diverse genera of the Proteobacteria.

Table 2. Plasmid stabilization modules present on IncP-1 plasmids isolated from wastewater treatment plants

<table>
<thead>
<tr>
<th>Plasmid(s)</th>
<th>System&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Genes</th>
<th>Gene products (classification)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Localization&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTB11, RK2</td>
<td>Par</td>
<td>incC-korB</td>
<td>IncC: ATPase (Pfam00991, COG1192, COG0455); KorB: ParB-like nuclease, transcriptional regulator (Pfam02195, COG1475)</td>
<td>Backbone</td>
<td>Active partitioning</td>
</tr>
<tr>
<td>Mrs</td>
<td>parCBA</td>
<td></td>
<td>ParA: resolvase, site-specific recombinase (Pfam00239, COG1961); ParB: nuclease (Pfam00565, COG1525)</td>
<td>Backbone</td>
<td>Multimer resolution</td>
</tr>
<tr>
<td>Psk</td>
<td>parDE</td>
<td>incC-korB</td>
<td>IncC: ATPase (Pfam00991, COG1192, COG0455); KorB: ParB-like nuclease, transcriptional regulator (Pfam02195, COG1475)</td>
<td>Backbone</td>
<td>Active partitioning</td>
</tr>
<tr>
<td></td>
<td>pSkl</td>
<td></td>
<td>KluA: transcriptional regulator (COG3609); KluB: Plasmid stabilization system protein (Pfam05016, COG3668)</td>
<td>Accessory module</td>
<td>Putative postsegregational killing</td>
</tr>
<tr>
<td>pB8</td>
<td>Psk (?)</td>
<td>orf3-parE</td>
<td>ParE: plasmid stabilization system protein (Pfam05016, COG3668)</td>
<td>Accessory module</td>
<td>Putative postsegregational killing</td>
</tr>
<tr>
<td>pB10, pJP4, pA81</td>
<td>Psk (?)</td>
<td>din-orf</td>
<td>Din: RelB antitoxin, DNA-damage-inducible protein (Pfam04221, COG3077); Orf: RelE addiction module toxin (Pfam03890, COG3041)</td>
<td>Backbone</td>
<td>Putative postsegregational killing</td>
</tr>
</tbody>
</table>

<sup>a</sup>Par, active partitioning system; Psk, postsegregational killing system; Mrs, multimer resolution system.
<sup>b</sup>Classification according to the Protein family database (Pfam), (Bateman et al., 2004).
<sup>c</sup>Classification according to the database ‘Clusters of Orthologous Groups of proteins (COG)’ which phylogenetically classifies proteins (Tatusov et al., 2001).
<sup>d</sup>Plasmid regions that do not belong to the core IncP-1-specific plasmid backbone are termed ‘accessory module’. (?) The postsegregational killing function has not yet been demonstrated on these plasmids.
(De Gelder et al., 2007). However, in the latter study pB10 was also found to be very or moderately unstable in a few strains that belong to species that are considered good hosts for IncP-1 plasmids. It thus remains to be investigated if the partitioning and possibly the PSK system function poorly or not at all in these strains. Plasmid pB4 only possesses din but the orf for the corresponding toxin was deleted during insertion of an accessory element. RelB/RelE family systems encoded by a din-orf region were also identified on the degradative IncP-1β plasmids pA81 (Jencova et al., 2004) and pJP4 (Trefaut et al., 2004). Interestingly, the IncP-1β plasmid pB8 contains two putative PSK-systems on accessory modules, namely KluA/KluB encoded upstream of klcABC, and Orf3/ParE of a Tn5501-related transposon that inserted downstream of the replication initiation gene trfA (Schlüter et al., 2005). The kluA-kluB region is considered as acquired module (Heuer et al., 2004) since it is only present on two other IncP-1 plasmids, namely R751 (Thorsted et al., 1998) and the degradative plasmid pADP-1 (Martinez et al., 2001). The role of KluAB in postsegregational killing has not been confirmed on any of these plasmids.

Multimer resolution systems (MRS) are widely spread among plasmids and resolve plasmid multimers that result from incorrect plasmid replication-termination or recombination between plasmid monomers. These MRS provide plasmid monomers that can be distributed among daughter cells during cell division, thus avoiding the formation of plasmid-free segregants. ParA of the IncP-1β MRS is a site-specific recombinase (resolvase) catalyzing resolution of plasmid-dimers at the resolution (res) site (Gerlitz et al., 1990; Summers et al., 1993b). The functions of the accessory MRS-components ParB (nuclease) and ParC are not precisely known. Genes encoding a putative MRS are present on the IncP-1β plasmid pTB11 (see Table 2). A relict of a multimer resolution system has so far only been found on the IncP-1β plasmids pB2/pB3, pRSB111, pJP4, pTP6 and pBPI36. They contain a complete parA gene downstream of the mating-pair-formation module trb, but it is currently not known whether the corresponding parA gene products are functional in multimer resolution. Interestingly, ParA of IncP-1β plasmids is only 77% identical to ParA of IncP-1β plasmids, which may suggest that plasmids of these subgroups acquired their MRS-modules independently.

In summary, stabilization systems not only ensure stable inheritance of plasmid replicons but also of plasmid-borne accessory modules that carry degradation or resistance determinants. Consequently, genetic determinants present on very stable plasmids will hardly get lost in many bacterial populations, even in the absence of selective pressure. Indeed, even though IncP-1 plasmids such as pB10 were shown to be unstable in a minority of hosts, they are known to be maintained over many generations in most of the strains that have been tested so far (De Gelder et al., 2007). The recently obtained sequence information of the WWTP plasmids suggests that most IncP-1 plasmids, including IncP-1β plasmids, encode more than one stabilization system. This may very well ensure plasmid stability in hosts where one of the mechanisms is not functional. The presence of such elaborate stabilization mechanisms emphasizes the challenge of ridding a bacterial population or mixed community of drug resistance determinants.

Conjugative transfer modules confer horizontal mobility to IncP-1 resistance plasmids

The most remarkable feature of IncP-1 plasmids is their high promiscuity, as they can conjugally transfer to and replicate in many phylogenetically distinct Gram-negative bacteria (Thomas & Smith, 1987). The complete apparatus necessary for conjugative DNA-transfer is encoded on the plasmid itself, by two transfer modules, termed Tra and Trb. The Tra module contains the genes traI to traC and traKLM, necessary for relaxosome formation, DNA-processing for transfer, initiation of transfer replication and guidance of the relaxosome complex to the secretion channel representing a type IV secretion system (Christie & Cascales, 2005, Christie et al., 2005). In addition, IncP-1α plasmids encode traB and traA of unknown function downstream of traC.

TraI, TraK, TraL and TraH are components of the relaxosome complex assembled at the origin of transfer (oriT) where the TraI relaxase catalyses single-strand nicking at the nick-site within oriT. Subsequently, rolling-circle transfer-replication possibly involving TraC-mediated priming is initiated. The relaxosome complex is then guided to the mating channel by the so-called coupling protein TraG (VirD4 homolog) which interacts with the cytoplasmatic face of the secretion apparatus (Pansegrau & Lanka, 1996; Adamczyk & Jagura-Burdzcy, 2003; Gomis-Rüth et al., 2004). Functions of other tra gene products are not precisely known.

Gene products encoded by the Trb core region (TrbB–TrbL) can be subdivided into three categories: (1) structural components of the sex-pilus and factors involved in processing of pilus subunits and pilus biogenesis, (2) channel components localized in the inner and outer membrane and (3) gene products involved in providing energy for the DNA-transport process and/or the biogenesis of pilus and channel components (Cascales & Christie, 2003; Christie, 2004). TrbC is the precursor of the major pilus subunit that is processed and cyclized by the serine protease (maturase) TraF and then assembled into pili (Kalkum et al., 2002, 2004). TrbF might be a minor pilus subunit or a chaperone involved in pilus assembly. Mature pili function in mediating first contact between donor and recipient bacterial cells. The genes trbB, D, E, F, G, H, I, J, and L encode components of the envelope-spanning secretion apparatus (Grahn et al., 2000; Cascales & Christie, 2003).
TrbB and TrbE most probably energize the DNA-transport process and/or biogenesis of the secretion system by NTP hydrolysis since these proteins possess NTP-binding motifs typically found in traffic NTPases (Krause et al., 2000). TrbN is a transglycosylase predicted to catalyse lysis of the peptidoglycan layer at the site where the secretion apparatus is assembled. TrbJ and TrbK are involved in entry exclusion, preventing uptake of an IncP-1 plasmid into a cell that already harbours an IncP-1 plasmid (Haase et al., 1996). The transcriptional regulator TrbA controls expression of the tra and trb operons (Zatyka et al., 2001). Precise functions of other trb gene products are currently unknown.

All IncP-1 plasmids analysed here harbour complete sets of tra and trb genes facilitating horizontal mobility of the plasmids and thereby of the plasmid-borne resistance modules. High levels of transfer activity were found for the IncP-1α plasmid pTB11 and the IncP-1β plasmids pB2/pB3 and pB10 whereas the IncP-1β plasmids pB4 and pB8 showed abnormally low conjugative transfer frequencies (Tauch et al., 2003; Schlüter et al., 2005). The reason for this behaviour could not be deduced from the nucleotide sequences of the latter two plasmids.

The high transfer efficiency of IncP-1 plasmids on surfaces such as on agar (Thatte et al., 1985) and possibly in biofilms, a common form of bacterial growth in nature and in clinical environments (Hausen & Wuerzt, 1999), may very well contribute to their persistence and widespread occurrence in natural and clinical habitats. As pointed out 30 years ago by Stewart and Levin (Stewart & Levin, 1977), in order for plasmids to persist in the absence of selection for the traits they encode, high transfer rates are required to compensate completely for occasional plasmid loss and slower growth rates of plasmid-containing vs. plasmid-free strains. A high horizontal transfer rate could thus ensure the persistence of plasmids, without the need for constant or fluctuating selection for plasmid-encoded traits, as also recently demonstrated through a newer statistical modelling approach (Ponciano et al., 2007). The key role of conjugative transfer in overall plasmid stability has also been empirically shown in liquid broth and on surfaces (Sia et al., 1995; Easter et al., 1997; Bahl et al., 2006) The question as to whether or not this horizontal spread can allow plasmids to persist as parasitic genetic elements without ever benefiting their host, is currently unanswered. Stewart and Levin (Stewart & Levin, 1977) and later studies (Bergstrom et al., 2000) reported that the transfer rates required for plasmid R1, measured in chemostats, were not high enough for the plasmid to be parasitic. Very recent results with IncP-1 plasmids on solid surface however suggest the opposite. The IncP-1 plasmid pJKK5 could completely invade a plasmid-free population of Esch. coli and Kluyvera sp. on agar surfaces in the absence of selection when the plasmid was initially present in 10% of the population, whereas a transfer-negative mutant was not able to invade (Bahl et al., 2006). Preliminary experiments with the IncP-1β plasmid pB10 on agar surface in the absence of antibiotics suggest that it can also invade an Esch. coli population under certain conditions, even when the initial fraction of plasmid-containing cells is extremely low (Fox and Top, unpublished data). Since these plasmids do not confer an obvious fitness benefit to these hosts under those conditions, these studies suggest parasitism of IncP-1 plasmids due to their efficient conjugative transfer in combination with high stability and low cost. Derivatives of IncP-1 plasmids such as pAI or pBP136 that do not code for any obvious beneficial trait could be used in the future to test the hypothesis of parasitism in IncP-1 plasmids. It has also been postulated that some conjugal plasmids without any obvious beneficial accessory functions may still benefit their host in habitats where attachment to surfaces is important for growth and/or survival, as genes required for pilus formation positively affect cell attachment and biofilm formation (Ghigo, 2001; Molin & Tolker-Nielsen, 2003; Reiner et al., 2006). Clearly knowledge on the importance of conjugative transfer for the persistence of IncP-1 plasmids in their natural environments is very limited and needs to be expanded through further plasmid ecology studies.

**IncP-1 plasmid functions are co-ordinately regulated to minimize the cost to their host**

IncP-1 plasmids encode regulatory elements facilitating coordinate control of plasmid functions such as vegetative replication, copy number control, incompatibility and plasmid-transfer. The regulatory network of IncP-1 plasmids has been studied in detail and was recently reviewed (Thomas, 2000a; Adamczyk & Jagura-Burzdzy, 2003). Therefore the description here is limited to some general considerations. IncP-1 plasmids encode four global repressor proteins (KorA, KorB, KorC and TrbA) that bind to corresponding operator motifs (Oα, Oβ, Oε and Oγ) in the vicinity of promoters located upstream of plasmid backbone operons. For example, the switch in gene expression between vegetative replication and conjugal transfer replication is accomplished by the action of KorA and KorB at the divergent promoters trfAp and trbAp upstream of the replication initiation operon sbb-trfA and the mating-pair-formation regulatory gene trbA, respectively (Jagura-Burzdzy & Thomas, 1994). Consensus KorA- and KorB-binding sites are associated with the promoters of sbb-trfA and trbA. Interplay of KorA and KorB facilitates effective and efficient coordination of vegetative plasmid replication and initiation of conjugal plasmid transfer.

It has been suggested that the IncP-1 plasmid regulatory network guarantees tight coordination of all plasmid functions at minimal costs for the host bacterium (Bingle &
Thomas, 2001; Thomas, 2006). Minimizing the negative effect on host fitness in the absence of selection, e.g. antibiotics, may benefit the competitiveness of the plasmid host, and therefore the persistence of the plasmid in bacterial communities. Indeed, even when a plasmid is perfectly stable, a high plasmid cost can be detrimental for plasmid persistence because the host(s) will be outcompeted by strains with no or less costly plasmids. Recent work indicates that plasmid pB10 confers a high cost to a few proteobacterial strains in laboratory broth cultures. Although the molecular basis of these poor host-plasmid associations is not yet unravelled, and low stability could in part be due to the specific growth conditions, these findings may suggest that the mechanism for ensuring low cost are not as promiscuous as previously thought (Heuer et al., 2006; De Gelder et al., 2007).

**IncP-1β antibiotic resistance and degradative plasmids evolved form a common ancestor**

Phylogenetic analyses revealed that IncP-1β antibiotic resistance and degradative plasmids have a common ancestor (Drøge et al., 2000; Schlüter et al., 2003; Heuer et al., 2004; Trefaut et al., 2004; Bahl et al., 2007). This becomes especially clear when comparing the genomic structure of the multiresistance plasmid pB10 to that of plasmid pJP4 from *Ralstonia eutropha* JMP134, which codes for degradation of chlorinated aromatic compounds (Trefaut et al., 2004). The backbone genes of the two plasmids are 99.9–100% identical to each other at the nucleotide sequence level, which is higher than the similarity of each plasmid with any other completely sequenced IncP-1β plasmid. Moreover, pB10 and pJP4 both carry a Tn501-type mercury resistance transposon that is inserted into exactly the same target site downstream of the replication initiation gene *trfA*. Likewise, it was already shown 20 years ago that pJP4 and the antibiotic resistance plasmids R906 and R772 carry a Tn501-like element at the same site (Smith & Thomas, 1987). Segments harbouring the Tn501-specific terminal inverted repeat IB_{merR} and the mercury resistance genes *merR-merTPADE-orf2’* are identical on pB10 and pJP4 (see Fig. 3). This clearly demonstrates that both plasmids have a recent common ancestor. While pJP4 must have evolved by acquisition of modules encoding degradative *tfld*-I and *tfld*-II genes and additional catabolic functions downstream of *trfA*, pB10 evolved through capturing two antibiotic resistance elements and a class 1 integron, respectively, downstream of *trfA* and between the conjugative transfer modules Tra and Trb. Moreover, plasmid pB10 was the first antibiotic resistance plasmid reported to contain the insertion sequence IS1071 (Schlüter et al., 2003), which usually is associated with degradative genes, such as on pJP4. Backbone sequences of other degradative IncP-1β plasmids such as pADP-1 [atrazine catabolism; (Martinez et al., 2001)], pUO1 [haloacetate catabolism; (Sota et al., 2003)], pTSA [p-toluenesulphonate degradation; (Tralau et al., 2001)] and pA81 [chlorobenzoic acid degradation; (Jencova et al., 2004)] were also found to be highly similar to the IncP-1β antibiotic resistance plasmids pB2, pB3, pB8 and pB10. Thus it appears that nearly identical IncP-1β backbone molecules carry either resistance or catabolic determinants depending on the selective pressure its host bacteria have encountered recently. It is very well conceivable that an IncP-1β degradative plasmid could serve as vehicle for the acquisition of antibiotic resistance modules.
under adequate selective conditions or vice versa that an antibiotic resistance plasmid might capture degradative traits and subsequently be selected in environments contaminated with organic pollutants. In this context it should be noted that recombination between two different IncP-1 plasmids temporarily coexisting in the same cell seems to be possible, as the rather weak surface exclusion between IncP-1 plasmids allows the cell to temporarily contain two different IncP-1 plasmids (Szpirer et al., 1999). This idea is supported by the fact that plasmids pB10 and pJP4 display a mosaic structure with respect to their backbone sequences which can easily be explained by genetic exchange between two different IncP-1β plasmids residing in the same cell (Schlüter et al., 2003). Although IncP-1 plasmids that carry both antibiotic resistance and catabolic genes have not been reported thus far, this could be simply due to lack of concurrent selection for these very different traits. WWTP could be potential sources of such a mixture of selection pressures, and therefore could be reservoirs of catabolic resistance plasmids. Thus, co-selection of drug resistance by chlorinated organics in the wastewater cannot be excluded and should be considered as an additional route in plasmid evolution and drug resistance spread.

**Accessory genetic modules on IncP-1 plasmids from wastewater treatment plant bacteria**

**Tn402-like transposons and their integrons**

Class 1 integrons are one of the five ‘historical’ classes of integrons that are found to be associated with mobile elements such as insertion sequences, transposons and conjugative plasmids (Mazel, 2006). They can be considered as naturally occurring cloning and expression elements that are able to incorporate and disseminate gene cassettes, which often contain antibiotic resistance genes (Bennett, 1999; Rowe-Magnus & Mazel, 2002; Fluit & Schmitz, 2004; Mazel, 2006). Class 1 integrons are associated with functional and nonfunctional Tn402-like transposons, and carry most of the known antibiotic-resistance gene cassettes (Mazel, 2006). However, integrons that are possibly not associated with Tn402-like transposition genes and resistance gene cassettes were recently recovered from different bacterial species residing in soil and sediments, which suggests that the dispersal of integrons may have begun before the ‘antibiotic era’ (Stokes et al., 2006). All integrons known today contain three key elements: (1) an intI1 gene encoding a site-specific integrase responsible for integration and excision of gene cassettes, which occurs at (2) the attI site, and (3) a promoter (P0) that drives expression of inserted gene cassettes. All gene cassettes generally consist of one gene and an imperfect inverted repeat at the 3’ end, called the attC site (or 59-base element), which varies in length from 57 to 141 bp and is the recognition site for the integrase (Mazel, 2006). Many class 1 integrons possess a conserved segment downstream of the gene cassette region, which is composed of the genes qacEA1, sulI and orf5 (see Fig. 5), encoding a small exporter protein that confers resistance to quaternary ammonium compounds, a dihydroteterate synthetase for sulphonamide resistance and a hypothetical protein of unknown function, respectively (Paulsen et al., 1993).

All IncP-1 antibiotic resistance plasmids described here contain class 1 integrons or at least a remnant of Tn402 in the case of plasmid pB4. The integrons of the IncP-1 plasmids pB2/pB3, pB8, pB10 and pTB11 are all found on Tn402-like transposons, and contain between one and three resistance gene cassettes, representing six different resistance determinants, in addition to the qacEA1 and sulI genes (see Fig. 5). Resistance genes located on integron gene cassettes are described in more detail below.

On the IncP-1β plasmids pB2/pB3, pB8 and pB10 the Tn402-like transposons carrying the integrons are inserted in the same intergenic region, between the Tra and Trb regions but into different target sites (Sota et al., 2007). In the IncP-1α plasmid pTB11 the Tn402-like transposon is located between the partitioning operons parDE and parC-BA. Plasmid pB3 contains two interleaved Tn402-like elements. The first one is a Tn402-remnant consisting of the resolvase gene trIC and the 25-bp terminal inverted repeats (IRI and IRt), (see Fig. 5c). The insertion site of the Tn402-remnant is located 412 bp downstream of traC and the 5-bp target site duplication of the element is still intact. Interestingly, the pB3 Tn402-remnant harbours a cassette-like element encoding a β-lactamase without recognizable attC-site. A similar arrangement was found on the IncP-1β plasmid pB4, which also contains a Tn402-remnant harbouring a cassette-like element conferring β-lactam resistance (Tauch et al., 2003). The Tn402-remnants of pB3 and pB4 share 83% identity at the nucleotide sequence level.

A Tn402-like transposon with integron on plasmid pB8 integrated 409 bp downstream of traC and did not leave traces of insertion (direct repeats), whereas on pB10 it is located 521 bp downstream of traC in the opposite orientation. A terminal inverted repeat could not be identified at one side of Tn402 on pB10 suggesting that a deletion removing also upp31.0 and the partition gene parA occurred in this region. The integrons of pB3, pB8 and pB10 all possess conserved segments consisting of qacEA1, sulI and orf5, but of those three only pB8 contains remnants of the Tn402-transposition module (intIAB). The integron on pB10 was shown to be functional since it was able to acquire an additional resistance gene cassette in situ (Szczepeanowski et al., 2004b).

On the IncP-1α resistance plasmid pTB11 the class 1 integron is part of a Tn402-like transposon that contains the
complete transposition module (*tniABQR*). Insertion of this element into the resolution site res-2 in the intergenic region between the partition operons *parCBA* and *parDE* caused 5-bp direct repeats (Tennstedt et al., 2005) suggesting insertion by transposition. Previous studies suggested that resolution sites for resolution of plasmid multimers are hotspots for integration of Tn402-like transposons, which means that IncP-1 plasmids are prominent target vehicles for the incorporation of integrons (Kamali-Moghaddam & Sundstrom, 2000; Partridge et al., 2002; Sota et al., 2007). The complete transposition module on pTB11 is nearly identical to the one present on the IncP-1β antibiotic resistance plasmid R751 (Thorsted et al., 1998). Since the pTB11 integron does not contain a conserved segment (*qacEA1-sul1* module) downstream of its gene cassette array it may either represent an ancestral form of integrons or have lost the segment over evolutionary time. In summary, the integrons described here were incorporated into these plasmids as part of Tn402-like transposons and contain different resistance gene cassettes. Gene cassette arrays can potentially be further modified by cassette uptake or loss.

It should be pointed out that integron resistance gene cassettes identical or almost identical to those present on the WWTP plasmids were previously identified in the genomes of different animal, human and plant pathogens and in nonpathogenic bacteria. This demonstrates that these microorganisms share a common pool of gene cassettes. It is thus very well possible that promiscuous, self-transmissible plasmids such as those of the IncP-1 group contribute to the dissemination of integron gene cassettes among phylogenetically distant pathogens and environmental bacteria. Moreover, integrons serve as platforms for the integration of additional resistance modules as exemplified by the integron of plasmid pB3, where the integrase gene *intI1* was disrupted by a composite tetracycline resistance transposon (Heuer et al., 2004). Extension of integron-based resistance regions via incorporation of other genetic elements encoding resistance determinants has been shown for other chromosomal and plasmid-borne integrons (Parkhill et al., 2001; Partridge & Hall, 2003; Szczeniowski et al., 2004a, 2005).

**β-Lactam resistance cassettes**

The β-lactams are among the most commonly prescribed antimicrobial drugs and represent one of the largest antibiotic classes. They target the bacterial cell wall biosynthesis and are used in the treatment of a variety of Gram-negative and Gram-positive infections. The most important resistance mechanism against β-lactams is expression of β-lactamas that cleave the amide bond of the β-lactam ring thereby inactivating the drug. β-Lactamas have been subdivided into four molecular classes, A–D, which include more than 300 different enzymes. Those displaying an extended substrate spectrum (ESBL–extended spectrum β-lactamas) are of particular clinical importance since they also inactivate the newer cephalosporins (Helfand & Bonomo, 2003; Poole, 2004a; Fisher et al., 2005). β-Lactamase genes are frequently found on integron-specific gene cassettes facilitating their dissemination among bacteria (Gootz, 2004; Weldhagen, 2004).

Plasmids pB8, pB10 and pTB11 contain *oxa* gene cassettes in their integrons, and so do the IncP-1 plasmids pSp14 and pSp33, which were also isolated from activated sludge bacteria but have not been completely sequenced (Tennstedt et al., 2003). These cassettes encode different class D β-lactamas of the oxacillinase type. The *oxa*2 gene cassettes present on pB8, pB10 and pTB11 are identical among each other and to *oxa*2 cassettes previously identified in the genomes of very different animal and human pathogens isolated in various countries, demonstrating their worldwide dissemination (Naas & Nordmann, 1999; Schlüter et al., 2003, 2005; Tennstedt et al., 2005). The *oxa*1 gene cassette of plasmid pSp33 is nearly identical to corresponding cassettes identified in different *Salmonella enterica* ssp. enterica clinical isolates from Korea (Lee et al., 2003; Lee et al., 2004). Plasmid pB4 contains the β-lactamase gene *bla* _NPS_ –1 on its Tn402 remnant (see above and Fig. 5c). The corresponding gene product is identical to group 2 d β-lactamase NPS-1 of plasmid pMLH50 from *P. aeruginosa* (Pai & Jacoby, 2001) and was classified as class D oxacillin-hydrolysing enzyme (OXA-type). It confers resistance to β-lactam antibiotics such as amoxicillin, azlocillin and piperacillin (Tauch et al., 2003). A similar enzyme, namely Bla _NPS_ –2 is encoded on the Tn402 remnant of plasmid pB3. This β-lactamase represents a new variant that is only 72% identical to Bla _BLCR_ –1 of plasmid p2293E from *P. aeruginosa* (Couture et al., 1992). This finding demonstrates that WWTP bacteria are a reservoir for β-lactamase genes that have not yet been isolated from clinical specimens. In addition, mobile integron gene cassettes seem to play an important role in the dissemination of β-lactamase genes in sewage habitats.

**Chloramphenicol resistance cassettes**

Chloramphenicol is a broad-spectrum antibiotic inhibiting protein synthesis through binding to the peptidyltransferase of the 50S ribosomal subunit. Chloramphenicol and some derivatives have been applied in human medicine, but due to a number of adverse effects they are only used nowadays for the therapy of a few life-threatening infections. Bacteria have developed different resistance mechanisms including inactivation of the drug by modification and active efflux (Wareham & Wilson, 2002; Balbi, 2004; Schwarz et al., 2004).
Chloramphenicol resistance (Cm\(R^\)) is encoded on plasmid pB3 as well as on pSp39, an incompletely sequenced IncP-1 plasmid isolated from an unknown sludge bacterium (Tennstedt et al., 2003). On pB3 the cmlA1 gene cassette, encoding a chloramphenicol efflux protein of the arabinose efflux permease family (COG2814), is inserted in the integron. Identical genes were previously identified in class 1 integrons of Klebsiella pneumoniae (Poirel et al., 2003) and a clinical strain of Esch. coli isolated from urine in China (AB212941). Plasmid pSp39 contains the catB2 integron cassette encoding chloramphenicol acetyltransferase CatB2. Very similar gene cassettes are present on pKBB958 from B. bronchiseptica (Kadlec et al., 2005), in the genome of Sal. enterica serotype enteritidis clinical isolates from southern Italy (Villa et al., 2002), on plasmid pMVH202 of Esch. coli and K. pneumoniae isolated in Spain (Törtola et al., 2005) and on plasmid pJR1 from an avian isolate of Pasteurella multocida (AY232670). More Cm\(R^\) gene cassettes are located on non-IncP-1 plasmids isolated from WWTP-bacteria (Tennstedt et al., 2003). Only very few plasmids isolated from wastewater bacteria confer chloramphenicol resistance, which might be due to the fact that chloramphenicol is no longer frequently used in human antimicrobial therapy.

**Sulphonamide and trimethoprim resistance**

Sulphonamides and trimethoprim show a bacteriostatic effect on microorganisms by inhibiting the folic acid metabolism (Huovinen et al., 1995; Huovinen, 2001; Sköld, 2001). Both antibiotics are used against a wide range of human pathogens (Huovinen et al., 1995; Huovinen, 2001; Chaowagul et al., 2005; Wuthiekanun et al., 2005). Trimethoprim and sulphonamides are also used for the treatment of bacterial infections in eel farms (Alcaide et al., 2005). Resistance is mainly due to horizontal acquisition of nonsensitive enzyme variants encoded on transposons and plasmids (Sköld, 2001).

Although genes encoding different dihydrofolate reductases and dihydropteroate synthetases were frequently found on plasmids and integrons isolated from WWTP bacteria (Tennstedt et al., 2003; Szczepanowski et al., 2004a, 2005), only sul and dfrII have been identified on IncP-1 plasmids analysed so far. As described above, the sul gene is part of the integron-specific 3′-conserved segment on plasmids pB2/pB3, pB8 and pB10 (Fig. 5). The trimethoprim resistance gene dfrII present on the IncP-1 plasmid pSp39 (Tennstedt et al., 2003) represents an integron-specific gene cassette (see Fig. 5). Identical genes were identified within integrons present on plasmids like pMVH202, found in clinical K. pneumoniae and Esch. coli isolates (Törtola et al., 2005) and pKBB958 (here named dfrB1) from the animal pathogen B. bronchiseptica (Kadlec et al., 2005). The IncP-1β prototype resistance plasmid R751 (Thorsted et al., 1998) also carries a dfrII gene cassette, which shares only 68% sequence identity with the one encoded on pSp39.

**Streptomycin resistance transposons and gene cassettes**

Streptomycin belongs to the class of aminoglycoside antibiotics and inhibits translation on the bacterial ribosome. Streptomycin was used for the treatment of several diseases such as tuberculosis and serious nosocomial infections (Davies & Wright, 1997). Moreover, streptomycin has been a major antibiotic in plant agriculture in the USA, for instance in the control of fire blight caused by Erwinia amylovora (McManus et al., 2002). Resistance against this antimicrobial drug is mainly due to acquisition of genes encoding aminoglycoside-modifying enzymes or alterations of the target-site.

All IncP-1 plasmids isolated from WWTP-bacteria and analysed at the genomic level confer streptomycin resistance (Sm\(R^\)), but this phenotype is encoded by different genetic determinants (see Figs 4 and 5). The IncP-1β plasmids pB4 and pB10 carry, respectively, a complete 5470 bp and a truncated variant of the Sm\(R^\) transposon Tn5393c (see Fig. 4). Tn5393c is a class II transposon (Tn3 family) and consists of the transposable gene tnpA, the resistase gene tnpR and the Sm\(R^\) genes strA and strB encoding aminoglycoside-3′-O-phosphotransferase and aminoglycoside-6-O-phosphotransferase. The 3′-part of tnpA was deleted on pB10 most probably by the insertion of IS1071. The insertion sites for Tn5393c are different on these two plasmids, i.e. downstream of the conjugative transfer operon traKLM on pB4, and downstream of trfA on pB10. Identical or almost identical copies of Tn5393 were recently identified on the resistance plasmid pRAS2 from the fish pathogen Aeromonas salmonicida ssp. salmonicida (L’Abée-Lund & Sørum, 2000), the conjugative plasmid pPSR1 from the phytopathogen P. syringae pv. syringae (Sundin et al., 2004), plasmid pEa34 from the phytopathogen Erw. amylovora (Chiou & Jones, 1993), the IncI1 plasmid R64 from the human pathogen Sal. enterica ssp. typhimurium (Accession no. NC_005014) and the multiresistance plasmid pTP10 from the opportunistic Gram-positive human pathogen Corynebacterium striatum (Tauch et al., 2000). A truncated Tn5393 derivative was also found to be inserted in the control region of an IncP-1β plasmid from a clinical isolate of Yersinia pestis, the causative agent of plague (Guivoule et al., 2001). All Tn5393 derivatives mentioned above are essentially identical with the exception that the elements present on pEa34, R64 and pTP10 are interrupted by insertion sequences or transposons.

Streptomycin used in the control of bacterial plant pathogens (McManus et al., 2002) and in plague treatment can explain the occurrence of Sm\(R^\) plant-associated bacteria and
Y. pestis strains, but the presence of a functional Tn5393-like transposon in the marine bacterium A. salmonicida ssp. salmonicida is less expected, since streptomycin has not been used in fish-farming environments. These findings illustrate the wide dissemination of the Sm\(^{R}\) transposon Tn5393 among human, animal and plant pathogens. Sundin \textit{et al.} (1995) even suggested that Tn5393 is indigenous to plant-associated and soil microbial communities with no prior exposure to streptomycin. The presence of Tn5393 derivatives on self-transmissible, broad-host-range IncP-1\(\beta\)
Genomics of IncP-1 plasmids from sewage treatment plants

Fig. 5. Transposons related to Tn402, integrons and integron gene cassette arrays identified on IncP-1 resistance plasmids residing in WWTP bacteria. (a) General structure of a typical resistance integron (‘class 1’). The integron consists of the integrase gene intI1, the promoters P\text{int} and P\text{attC} and the integron-specific attachment site (attI). Gene cassettes can be integrated into the integron structure via site-specific recombination at attI and attC (recombination site of gene cassettes). Many class 1 integrons contain a conserved segment downstream of the gene cassette region composed of the quaternary ammonium compound resistance gene qacE\text{A}1, the sulphonamide resistance gene sul and orfS of unknown function. (b) Integrons identified on Tn402-like transposons in the plasmids R751, pB3, pB8, pB10 and pTB11. The colour code for integron segments is identical to the one used in Fig. 4a. Transposition modules (tmr) of Tn402 are indicated in blue. The triangle designated Tn-tet marks the insertion of a composite tetracycline resistance transposon in pB3 intI1. White circles mark target-site duplications of Tn402-like transposon insertions (DR, direct repeats). (c) Plasmids pB3 and pB4 carry similar Tn402-remnants consisting of tmrC and cassette-like elements encoding, respectively, \textit{bla}_{NPS-2} or \textit{bla}_{NPS-1} for β-lactamases. (d) Integron gene cassette arrays located on IncP-1 plasmids isolated from WWTP bacteria. In contrast to the completely sequenced plasmids shown in (b) and (c), only the integron gene cassette arrays were sequenced for the plasmids shown in (d) (Tennstedt et al., 2003).

Fig. 4. Resistance modules identified on IncP-1 plasmids from WWTP bacteria. Genes for transposition functions of insertion sequences (IS) or transposons (Tn) are marked in red. Red rectangles symbolize terminal inverted repeats (IR) of IS elements and transposons, respectively. Triangles indicate insertions of additional mobile genetic elements. (a) Drug-resistance modules. Plasmids pTB11, pB10 and pB3 carry, respectively, a tetracycline resistance (tet) region, a derivative of the tetracycline resistance transposon Tn1721, and a composite tetracycline transposon carrying \textit{tetA(C)}-\textit{tetR(C)}. Plasmids pB4 and pB10 contain derivatives of the \textit{strA8} streptomycin resistance transposon Tn5392c. The Tn5392c derivative on pB10 is truncated (indicated by a vertical line). The kanamycin resistance module orf3-\textit{aphA}4 on pTB11 is not flanked by transposition modules. Plasmid pRS8111 contains a macrolide resistance module (\textit{mph}) that is flanked by two IS elements. (b) Modules encoding heavy metal resistance, multidrug efflux and resistance to quaternary ammonium compounds. Plasmid pB4 encodes a tripartite MDR efflux system (Reg—regulator, MFP—membrane fusion protein, RND—resistance-nodulation-division type permease, OMF—outer membrane factor). A mercury (mer) resistance transposon related to Tn501 was found on pB10. Plasmid pB4 harbours the complete chromate (\textit{chr}) resistance transposon Tn5719. The Tn5501-like transposon on pB8 mediates resistance to quaternary ammonium compounds through \textit{qacF}. (c) ‘Cryptic’ transposon on pB8, encoding two orfs of unknown function.

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plasmids residing in WWTP will further contribute to the spread of streptomycin resistance among environmental bacteria.

The IncP-1 plasmids pB3, pB8, pTB11, pSm47, pSp14, pSp33 and pSp39 (Tennstedt et al., 2003) contain different variants of the integron aadA gene cassette (aadA1, aadA2, aadA4 and aadA5; see Fig. 5) which encode streptomycin/ spectinomycin-3′′-O-adenyllytransferases conferring resistance to the aminoglycosides streptomycin and spectinomycin. The aadA2 gene cassette for instance has also been identified in integrons of many other bacteria that belong to at least 11 genera (Shaw et al., 1993). Likewise, wide dissemination has been observed for the gene cassettes aadA1, aadA4 and aadA5, demonstrating that different environmental and pathogenic bacteria share a common pool of integron-specific resistance gene cassettes (Tennstedt et al., 2003).

Other aminoglycoside resistance modules and cassettes

Aminoglycosides are among the most commonly used broad-spectrum antibiotics. Resistance is mainly conferred by enzymes that modify aminoglycosides through phosphorylation, adenylation or acetylation (Migeotte-Leclercq et al., 1999; Kotra et al., 2000).

The IncP-1α plasmid pTB11 carries the apha-orfβ module encoding aminoglycoside-3′′′-phosphotransferase and a putative adenylyltransferase (pam04439) of unknown function. This module is not part of a mobile element and the mechanism by which it entered the plasmid is still unknown (Tennstedt et al., 2005). The pTB11 apha gene shows the highest degree of similarity to the corresponding gene of plasmid RP4 which originates from a clinical isolate (Datta et al., 1971; Ingram et al., 1973).

Other integron-specific aminoglycoside resistance gene cassettes are located on pTB11 and on the partially sequenced IncP-1 plasmid pSP32 (Tennstedt et al., 2003) (see Fig. 5). The pTB11 aacA4 cassette encodes an aminoglycoside-6′-N-acetylation transfering resistance to different aminoglycosides such as tobramycin, amikacin and gentamicin. The integron of plasmid pSP32 contains two aminoglycoside resistance gene cassettes, aadB and aacA29b, which code for an aminoglycoside-2′′′-O-adenyllytransferase (AadB) and an aminoglycoside-6′-N-acetyltransferase (AacA29b). AadB confers resistance to gentamicin, tobramycin and kanamycin and is also encoded on a class 1 integron of a clinical Acinetobacter baumannii isolate from a hospital in South Africa (AF364344). The aacA29b cassette represents a new variant of aacA29u which was previously described to be located in In59 of a clinical P. aeruginosa isolate (Poirel et al., 2001). Sewage bacteria thus harbour integrons that are either widely disseminated or have not yet been identified in other bacteria.

Tetracycline resistance transposons and modules

Tetracyclines inhibit prokaryotic protein biosynthesis by preventing attachment of aminocyl-tRNA to the ribosome and are thus active against a wide variety of Gram-negative and Gram-positive pathogens. Due to increasing resistance to tetracyclines these drugs are used less frequently as a first choice. Current indications for tetracycline prescriptions include brucellosis, rickettsial infections, tularemia, Lyme disease and typhus. Mechanisms of resistance include active efflux, ribosome protection, target-site alterations and reduction of cell permeability (Roberts, 1996, 2005; Chopra & Roberts, 2001).

Three completely sequenced IncP-1 plasmids from WWTP bacteria mediate resistance to tetracycline, encoded by two different resistance determinants (see Fig. 4). Plasmid pB10 possesses a 6388-bp remnant of the tetracycline resistance (TcR) transposon Tn1721, which is part of a complex resistance region downstream of the replication initiation gene tfrA. Tn1721 was first described in Esch. coli and consists of orfI encoding a methyl-accepting chemotaxis protein, the transposition module trpR-trpA, the TcR genes tetR and tetA and a truncated transposase gene *trpA* (Allmeier et al., 1992). The element is flanked by 38-bp inverted repeats. An internal third inverted repeat, termed IIR, is located downstream of tetR. TetA is a membrane bound efflux protein of the major facilitator superfamily (MFS) exporting tetracycline from the cell. In pB10 the orfI and part of the left-hand-side transposition module of the Tn1721 derivative were deleted. A pecM-like gene encoding a putative integral membrane protein has been identified downstream of tetA and is present on all Tn1721 derivatives analysed so far. Nearly identical Tn1721 derivatives were found on the conjugative IncU plasmids pRAS1 from the fish pathogen Aer. salmonicida (Sorum et al., 2003) and pFBAT6 from Aer. punctata isolated from hospital effluent (Rhodes et al., 2004), the R plasmid pSC138 from the human pathogen Sal. enterica ssp. enterica serovar cholerasuis (Chiu et al., 2005), the R-plasmid pFPTB1 from Sal. enterica ssp. enterica serovar typhimurium (Pasquali et al., 2005), the conjugative R plasmid pC15-1a from a clinical Esch. coli isolate (Boyd et al., 2004) and in the genome of the opportunistic pathogen Ac. baumannii (Ribera et al., 2003). Moreover, distribution of Tn1721 derivatives could be demonstrated for phyloplane bacteria in Michigan apple orchards (Schnabel & Jones, 1999). These findings provide evidence that there is genetic exchange between bacteria of the human-, animal- and plant-associated environmental compartments. Tetracyclines have been used in large amounts in aquaculture to control fish diseases, in agriculture to protect plants from plant pathogens and in veterinary medicine and animal growth promotion (Chopra & Roberts, 2001). Thus, it is not astonishing that bacteria
harbouring mobile plasmids with Tn1721-specific tet genes are found in wastewater purification plants, from where they can transfer further in the environment.

The central core of Tn1721 was also identified on the IncP-1\(\alpha\) plasmids RP4 (Pansegrau et al., 1994) and pTB11 (Tennstedt et al., 2005) (see Fig. 4). In contrast to RP4, plasmid pTB11 contains pecM and pncA (encoding an amidase/amido-hydrolase related to nicotinamidase) downstream of tetA.

The IncP-1\(\beta\) plasmid pB3 harbours a 5385-bp composite tetracycline (tet) resistance transposon inserted into the integrase gene intI1 of the integron. The presence of 8-bp target site duplications indicates insertion by transposition. The pB3 tet-transposon is composed of the Tc\(^R\) genes tetA(C) (tetracycline efflux protein) and tetR(C) (tetracycline repressor) that are flanked by copies of the insertion sequence IS26. TetA(C) is only 86% similar to TetA from pB10 and therefore belongs to another class of tetracycline efflux proteins. A very similar tet-transposon is present on Tn1404 of Pseudomonas sp. R9 isolated from a Michigan apple orchard (Schnabel & Jones, 1999). Conservation even includes the insertion-site of the tet-transposon in intI1, but because only parts of Tn1404 were sequenced further comparison is not possible. The frequent occurrence of tetracycline in farming plant pathogens might have selected for Tc\(^R\) soil and phytosphere bacteria. The Tc\(^R\) module tetA(C)-tetR(C) was also found on pRAS3 of the fish pathogen Aer. salmonicida (L'Abée-Lund & Sørum, 2002), the conjugative broad-host-range IncN plasmid R46 of Sal. enterica ssp. typhimurium ([Brown & Willetts, 1981] AY046276), the R-plasmid pSC101 of Salmonella sp. (Bernardi & Bernardi, 1984) and on a genomic island located in the chromosome of the pig pathogen Chlamydia suis (Dugan et al., 2004). It is worth noting that veterinary chlamydial infections are often treated with antimicrobial compounds of the tetracycline family (Chopra & Roberts, 2001). In summary, nearly identical tetA(C)-tetR(C) modules were identified in plant-associated bacteria, human and animal pathogens and in noncharacterized bacteria from a WWTP.

**The IncP-1\(\beta\) plasmid pB4 encodes a multidrug efflux system**

Efflux-mediated multiresistance becomes a problem of increasing relevance for the treatment of infectious diseases. Apart from drug-specific efflux mechanisms, systems able to export multiple antimicrobials contribute to intrinsic and acquired multidrug resistance (MDR). RND-type (resistance, nodulation, division) transporters consisting of a permease, a periplasmic membrane fusion protein (MFP) and an outer membrane factor (OMF) are most commonly encoded in the chromosome of bacteria. In contrast, plasmid-borne RND-type MDR systems have rarely been identified so far (Li & Nikaido, 2004; Poole, 2004b, 2005).

The IncP-1\(\beta\) plasmid pB4 contains a segment of foreign DNA encoding a tripartite multidrug efflux system downstream of klcA. On the left-hand side, this region is flanked by the din gene for a putative DNA-damage-inducible protein (RelB, COG3077; RelB antitoxin, pfam04221). Interestingly, a din gene is clustered with genes for, respectively, a cat ion efflux system protein and a component of a multidrug efflux system in the genome of the phytopathogen X. fastidiosa (Simpson et al., 2000). The pB4 multidrug efflux system is composed of an AcrA-type membrane fusion protein (AcrA, COG0845), an AcrB-type cation/ multidrug efflux pump spanning the inner membrane (AcrB, COG0841, pfam00873) and an outer membrane efflux protein (OEP, pfam02321). In addition, the regulatory gene nfsB was identified upstream of the MDR gene cluster on pB4. Functional analysis revealed that the pB4 multidrug efflux system confers high-level erythromycin and roxithromycin resistance to the host bacterium Pseudomonas sp. B13 (Tauch et al., 2003). These agents are clinically important antibiotics that inhibit protein biosynthesis at the large subunit (50S) of ribosomes (Blondeau, 2002; Blondeau et al., 2002). Erythromycin, for example, is frequently used to treat infections caused by bacteria belonging to the genera Streptococcus, Listeria, Legionella, Bordetella, Mycoplasma, Chlamydia and Campylobacter.

The components of the pB4 multidrug efflux system show the highest similarity to the products of the mexCD-oprJ determinant encoded in the P. aeruginosa chromosome (Tauch et al., 2003), but homologous systems were also found in the phytopathogens P. syringae pv. tomato (Buell et al., 2003) and pv. syringae (Feil et al., 2005) and in the saprophytic soil bacteria P. fluorescens (Paulsen et al., 2005) and P. putida (Nelson et al., 2002). On the right-hand side the pB4 multidrug efflux region is flanked by a new IS element of the IS21 family. Currently it is unknown whether this IS element played a role in the acquisition of the multidrug efflux region. The pB4 efflux transporter contributes to acquired resistance against clinically relevant antibiotics and due to the mobility of the plasmid it is very likely that such resistance determinants can be further disseminated among bacteria.

Another plasmid, namely pRSB101, from a WWTP bacterium also encodes a tripartite multidrug efflux system. In contrast to the pB4 system the pRSB101 MDR complex is composed of an AcrA-like MFP, (COG0845, pfam00529), an ATP-binding-cassette-type (ABC) permease component (COG5077, pfam02687) and an ABC-type ATPase component (COG1136, pfam00005), (Szczepanowski et al., 2004a).

It is conceivable that the presence of plasmid-borne multidrug efflux systems conferred an adaptive advantage to bacteria before the antibiotic era, for example because of
low-level resistance to plant-borne or other antimicrobial compounds. A recent study has indicated that homologues of multidrug resistance genes such as mexF are required for fitness of Shewanella oneidensis MR-1 in anoxic aquifer sediments (Groh et al., 2007). Thus, plasmid-encoded multidrug resistance determinants might have facilitated initial survival of the host and provided the opportunity to incorporate additional resistance genes.

**Resistance determinants to quaternary ammonium compounds and disinfectants**

Resistance to biocides (antiseptics and disinfectants) is of particular importance especially with respect to clinical hygiene. Quaternary ammonium compounds (QACs) such as cetrimide and benzalkonium chloride are amphoteric surfactants that are widely used as antiseptics and disinfectants for clinical purposes, in consumer products and in industrial environments (McDonnell & Russell, 1999; Gilbert & McBain, 2003). QAC resistance is mainly due to qac genes encoding small multidrug resistance (SMR) proteins mediating proton-dependent efflux of these compounds from the cell (Russell, 1998). QAC resistance genes have been found in a broad range of Gram-negative and Gram-positive species from clinical and environmental sources and are often integral elements of integrons that also contain antibiotic resistance genes (Paulsen et al., 1993; Kazama et al., 1998a, b). In a recent study it could be demonstrated that QAC selection in a natural environment co-selects for antibiotic resistance mediated by integron-specific resistance gene cassettes (Gaze et al., 2005).

The IncP-1β plasmid pB8 contains a 5837-bp QAC resistance transposon of the Tn3 family (class II transposons) that inserted in the accessory region downstream of trfA. This transposon harbours a putative plasmid stabilisation system module (see Table 2) and a quaternary ammonium compound resistance region consisting of the genes orf4 for an AcrR/TetR-type regulator and qacF encoding a SMR protein. The origin of the latter module and the mechanism by which it was integrated into the transposon structure are currently unknown. The qacF region mediates resistance to cetyltrimethylammonium bromide (CTAB) and benzalkonium chloride (Schlüter et al., 2005).

Other qac genes were identified on integron-specific gene cassettes (qacF and qacH) and in the 3′-conserved segment of class 1 integrons (qacEDA1) on plasmids from WWTP bacteria [see Fig. 5; (Tennstedt et al., 2003)]. Since these qac genes are linked to antibiotic resistance genes it is very likely that presence of quaternary ammonium compounds selects for the persistence and further spread of antibiotic resistance determinants and **vice versa**. Some authors even speculate that uncontrolled, intensive use of antiseptics and disinfectants poses a risk of increasing widespread occurrence of antibiotic resistance (Gaze et al., 2005).

**Mercury resistance transposons**

Transposons carrying mercury resistance determinants (mer) are widely distributed in clinical and environmental bacteria (Misra, 1992; Mindlin et al., 2001). Mercury naturally exists in small amounts in the environment and is cycled by both geological and biological processes. Additional mercury contamination comes from human activities such as use of mercurial fungicides in agriculture, disinfectants in medical practice and mercury catalysts in industry (Osborn et al., 1997; Kümmere, 2001; Barkay et al., 2003). Another source of mercury is dental amalgam fillings, and it has been shown that mercury released from amalgam can select for both mercury- and antibiotic resistant oral and intestinal bacteria since mercury resistance determinants are often linked to antibiotic resistance genes on the same mobile element (Summers et al., 1993a; Davis et al., 2005).

Plasmid pB10 carries a truncated narrow spectrum mer resistance transposon that inserted downstream of trfA (see Fig. 4). The mer gene cluster encodes a regulatory protein (MerR), a mercuric ion transport protein (MerT) and a periplasmic mercuric ion binding protein (MerP), mercuric reductase (MerA), a secondary regulatory protein (MerD) and proteins of unknown function (MerE and Orf-2). The pB10 mer cluster is nearly identical to corresponding operons present on Tn4378 of the Cupriavidus (formerly Ralstonia) metallidurans CH34 megaplasmid pMOL28 (Taghavi et al., 1997), on Tn501 of the P. aeruginosa plasmid pVS1 (Brown et al., 1986), the Shigella flexneri virulence plasmid pWR501 (Venkatesan et al., 2001) and the P. stutzeri mercury resistance plasmid pPB (Reniero et al., 1998), and on the Tn501 remnant on pJP4 (Trefaut et al., 2004).

Plasmid pB8 only contains a mer transposon remnant that inserted downstream of trfA (see Fig. 4). This 1989 bp accessory element related to Tn501/Tn21-like mercury resistance transposons is bordered by, respectively, 38 and 30 bp inverted repeats. Only the 3′-end of the mer regulatory gene merR has been retained. The accessory genes carried by this mobile element encode an uncharacterized conserved protein that contains double stranded β-helix domains (COG1917) and a LysR-type transcriptional regulator. A nearly identical element containing two additional IS4321 copies is present on the prototype IncP-1β plasmid R751 (Thorstedt et al., 1998). The origin and function of the pB8 Tn501/Tn21-like element are unknown.

Mercury resistance (Hg⁰) regions have been frequently found on IncP-1β plasmids. For example the degradative plasmids pJP4 (Trefaut et al., 2004), pADP-1 (Martinez et al., 2001) and pUO1 (Sota et al., 2003) contain truncated or complete Hg⁰ transposons, whereas the antibiotic
resistance plasmid R751 only possesses remnants of a HgR transposon (Smith & Thomas, 1987; Thorsted et al., 1998). Only recently the first example of an IncP-1β plasmid (pTP6) that encodes HgR, but does not carry any antibiotic resistance or degradation genes, was isolated from mercury-polluted river sediments in Kazakhstan and completely sequenced (Smalla et al., 2006). The frequent occurrence of mercury transposable elements on IncP-1 plasmids, together with drug resistance determinants or with catabolic determinants, is striking and suggests that (1) the mer genes were one of the first accessory genes acquired by IncP-1β plasmids (Smith & Thomas, 1987), or (2) they are often selected because of the global presence of mercuric chloride in clinical and environmental habitats.

**Chromate resistance determinants**

Chromium input into the biosphere mainly results from industrial processes such as alloy fabrication, leather tanning, production of pigments and electropating. Chromium is usually associated with oxygen as chromate (CrO$_4^{2-}$) or dichromate (Cr$_2$O$_7^{2-}$) and is highly toxic for microorganisms since it affects several biological processes (Cervantes et al., 2001). Due to the prevalent selection pressure, different chromate-resistant bacteria were isolated from the environment and clinical settings (Nies, 2003). Chromate resistance determinants are often carried on plasmids (Cervantes & Silver, 1992).

The IncP-1β plasmid pB4 carries the 8355 bp Tn5719 chromate resistance (Chr$^R$) transposon downstream of trpA. This element encodes a conserved hypothetical protein, an uncharacterized conserved protein (COG4275), a superoxide dismutase (SodA, COG1060, pfam02777), a chromate transporter (ChrA, COG2059, pfam02417) and another uncharacterized conserved protein (COG4275). The transposon is completed by a Tn3-related transposition module tnpR-tnpA encoding, respectively, a resolvase and a transposase. However, no resistance to chromate was detectable in *Pseudomonas* sp. B13 and *Esch. coli* DH5α harbouring pB4 (Tauch et al., 2003). The original pB4 host bacterium is not known since pB4 was obtained by exogenous plasmid isolation. Chr$^R$ determinants were previously identified on the conjugative plasmid pUM505 from a *P. aeruginosa* clinical strain (Cervantes et al., 1990) and the heavy-metal resistance plasmid pMOL28 from *Cup. metallidurans* (Margey et al., 2003). Plasmid pMOL28 carries the genes chrB, chrA, chrC, chrE and chrF that are orthologous to corresponding genes on pB4 Tn5719 with the exception of chrE encoding a sulfurtransferase (COG0607). A chrE-like gene is missing on Tn5719. It should be noted that the pMOL28 chromate resistance module is not flanked by any transposition genes. Plasmid pUM505 only contains a functional chrA gene that is necessary and sufficient for chromate resistance (Cervantes et al., 1990). The Chr$^R$ element on pUM505 most probably is a deletion derivative of Tn5719, which would make Tn5719 on pB4 the first complete Chr$^R$ transposon described.

On plasmids other than pB4, Chr$^R$ genes have also sometimes been found linked to antibiotic resistance genes, which suggests that, just like HgCl$_2$, the presence of chromate may select for the maintenance of antibiotic resistance determinants. For example, the antibiotic resistance plasmids pRBDHA from *K. pneumoniae* (Verdet et al., 2006) and pU302L from *Sal. enterica* ss. enterica serovar typhimurium (AY333434) both encode chromate transporter genes.

**Bacteria carrying IncP-1 resistance plasmids are released with the final effluents of the wastewater treatment plant**

Several lines of evidence indicate that bacteria harbouring IncP-1 antibiotic resistance plasmids are released from WWTP into surface waters. First, different IncP-1 plasmids could be isolated by the exogenous isolation method from effluent samples of WWTP (Tennstedt et al., 2003, 2005), and some of these plasmids contain integron-specific sequences and resistance gene cassettes. Other studies describe the identification of resistance determinants in bacteria from habitats downstream of WWTP (Smalla & Sobecky, 2002).

Thus it is very likely that IncP-1 plasmid-borne resistance regions are extended, recombined, modified, exchanged and disseminated in and among bacteria residing in habitats downstream of WWTP. Plasmids with new resistance properties could be transferred to human pathogens, thus increasing the difficulties of treating infectious diseases, including nosocomial infections.

**The relative importance of IncP-1 plasmids for drug resistance spread in wastewater treatment plants**

This overview of antibiotic resistance IncP-1 plasmids from WWTP may generate the impression that these particular plasmids are (1) abundant in WWTP, (2) typically found in WWTP, or (3) the major shuttle vectors for drug resistance and degradation of chlorinated organic compounds. Before making any such assumptions, the results from different studies, habitats, and plasmid isolation methods must be compared, and the potential bias for or against Inc-P-1 plasmids for each of these methods must be considered. They are summarized here, but the reader is referred to a recent review (Smalla & Sobecky, 2002) for a more detailed overview, which also includes direct detection of plasmid DNA by hybridization, not discussed here.
Traditionally, studies on the genetic diversity of plasmids required that plasmid DNA be extracted from hosts that could be cultured. Thus until two decades ago all known plasmids were detected in bacteria that were first isolated from a variety of habitats as pure cultures, usually based on a specific phenotypic trait (virulence, resistance to or degradation of various compounds, etc.). This method was called ‘endogenous plasmid isolation’. Of the 19 IncP-1 plasmids for which the complete genomes are available in GenBank, 10 have been isolated using this approach. They were found to encode degradation of chloro-organics (pJP4, pEST4011, pUO1, pADP-1, pA81) resistance to antibiotics (RP4, pBS228, R751), or no known phenotypic traits (pA1 and pBP136). Although IncP-1 plasmids can be found with this approach, in some studies none of the many characterized plasmids belonged to the IncP-1 group (Kobayshi & Bailey, 1994; Sobecky et al., 1997, 1988). Based on this nonsystematic and nonquantitative collection of data, not much can be concluded about the relative abundance of IncP-1 plasmids in different habitats. Moreover, this endogenous plasmid isolation approach almost certainly provides a distorted view of plasmid diversity, given that a large proportion of bacterial species have not yet been cultured (Pace, 1997).

In an attempt to circumvent the cultivation bias so-called ‘exogenous plasmid isolation’ methods were developed by J. Fry and coworkers 20 years ago (Bale et al., 1987, 1988). The first method, ‘biparental exogenous plasmid isolation’ relies on the conjugal transfer of a plasmid from an indigenous population to a suitable recipient strain, followed by selection for recipient cells that have acquired a specific phenotypic trait thought to be plasmid encoded. The main advantages of this method are its direct selection for transferable plasmids and its independence from culturing plasmid hosts. This approach has been successfully used by several groups to isolate a wide diversity of plasmids that encode HgR or antibiotic resistance, or the ability to degrade a specific organic compound, from various environments (Bale et al., 1987, 1988; Fry & Day, 1990; Top et al., 1995; Lilley et al., 1996; Dahlberg et al., 1997; Dronen et al., 1998, 1999; Smit et al., 1998; Stuart-Keil et al., 1998; Dröge et al., 2000; Schneiker et al., 2001; Heuer et al., 2002; van Overbeek et al., 2002; Smalla et al., 2006; Ono et al., 2007). All of the six plasmids from WWTP described in detail in this review, and pKJK5 from barley rhizosphere (Bah et al., 2007), were obtained using this method with selection on antibiotic resistance. Surprisingly, very large percentages (c. 50–100%) of the antibiotic resistance plasmids that were captured from WWTP in various recipient hosts, are IncP-1 plasmids (Dröge et al., 2000; Heuer et al., 2002; van Overbeek et al., 2002; Tennstedt et al., 2005). When that same approach was used on manured soil samples, IncP-1 plasmids were found less frequently (Smalla et al., 2000; Heuer & Smalla, 2007) (K. Smalla, pers. commun.). Moreover, when HgR plasmids were captured from rivers, marine environments, soils, rhizospheres and phyllospheres, the majority of the plasmids did not belong to the IncP-1 group, but to so far uncharacterized plasmid groups (Bale et al., 1987; Lilley et al., 1994, 1996; Dahlberg et al., 1997; Dronen et al., 1998, 1999; Smit et al., 1998; Schneiker et al., 2001; Smalla et al., 2006). These findings strongly suggest that IncP-1 plasmids may be particularly abundant in WWTP, and thus play a critical role in the spread of antibiotic resistance genes in these habitats. However, there are many possible sources of bias in these studies that may favour the isolation of IncP-1 plasmids, such as the high transferability of IncP-1 plasmids on rich agar media, their high stability and low cost in the recipient strains used, etc. Moreover, these results are based on slightly different methods in different laboratories, and a systematic, statistically valid, large-scale comparative analysis of plasmid abundance in various habitats does not exist.

Another interesting observation is the dearth of IncP-1 plasmids among those isolated using the third method, ‘triparental exogenous plasmid isolation’. This method was developed in order to isolate self-transmissible plasmids without the bias of a specific phenotypic marker (Hill et al., 1992; Top et al., 1994). It involves a mating with (1) a donor that carries a nonself-transmissible, mobilizable vector with selectable markers; (2) a selectable recipient strain; and (3) the indigenous populations of a habitat. Recipient cells that have acquired the mobilizable plasmid are then selected, and these have often simultaneously acquired self-transferable plasmid(s) from the indigenous community (Top et al., 1994; van Elsas et al., 1998). While two of the 18 sequenced IncP-1 plasmids, pTP6 and pQKH54 were isolated with this method from Hg-polluted soil and river epilithon (Haines et al., 2006; Smalla et al., 2006), other plasmids isolated this way from WWTP, river epilithon, soil and rhizospheres usually have a broad host-range but rarely belong to the IncP-1 group (Top et al., 1994; Hill et al., 1995; Dronen et al., 1998; van Elsas et al., 1998; Tauch et al., 2002; G tstalder et al., 2003; Smalla et al., 2006). Here too, the frequency distribution of plasmid types among transconjugants does not necessarily reflect their true distribution in nature because, for example, the most abundant transferable BHR plasmids might not mobilize the vector very well under laboratory conditions, or might be unstable in the recipients, and would thus not be easily captured. However, since IncP-1 plasmids are known to mobilize the vectors used in these studies (IncQ) extremely well, and are very stable in the recipients used, they should have been captured more frequently if they were the most abundant mobilizing plasmids in these habitats.

In conclusion, non-IncP-1 BHR plasmids seem generally more prevalent than IncP-1 plasmids in several habitats including WWTP. However, in spite of the many caveats in
the studies performed so far, among drug resistance BHR plasmids in WWTP, those of the IncP-1 group seem to be most abundant and/or most involved in the rapid spread of resistance determinants between distantly related bacteria. This observation, in combination with the frequent association of IncP-1 plasmids with pathways for degradation of man-made compounds (Top & Springael, 2003), may also suggest that these mobile elements are particularly selected as tools for rapid adaptation of bacterial communities to recent anthropogenic changes in their environment. More rigorous quantitative ecological studies, combined with large-scale plasmid genome comparisons, possibly including metagenomic approaches, will greatly increase the insight into the relative importance of the various plasmids in the spread of resistance in WWTP.

Conclusions

The following important insights were derived from genomic analysis of a set of IncP-1 resistance plasmids from WWTP bacteria. (1) They encode modules mediating resistance to almost all clinically relevant antimicrobial drug classes including tetracyclines, aminoglycosides, macrolides, β-lactams, chloramphenicol, sulphonamides and trimethoprim. In addition, genes were identified that encode resistance to heavy metals (mercury and chrome) or quaternary ammonium compounds (used in detergents). (2) All IncP-1 plasmids sequenced so far are modularly composed of backbone segments encoding essential plasmid functions, and when accessory mobile genetic elements are present, they are always inserted in one, two or (maximum) three specific regions. This confirms the 20-year-old observation of the conserved backbone and modular structure of IncP-1 plasmids based on restriction mapping and heteroduplex analysis (Smith & Thomas, 1987, 1989). These plasmids undoubtedly form a modular flexible, dynamic and adaptive genetic structure. (3) The highly conserved backbone in contrast with the enormous diversity of accessory genes strongly suggests that the plasmid backbones serve as efficient vehicles for shuttling modules encoding antibiotic, heavy metal, and disinfectant resistance, degradative capabilities and/or other accessory functions. Since combinations of antibiotic and heavy metal resistance genes were identified on a single plasmid, such plasmids could be beneficial to their host and therefore persist in the absence of antimicrobials. (4) IncP-1 plasmids often carry more than one stabilization module, which may contribute to the very stable inheritance of these plasmids and their accessory elements in a broad range of bacterial populations. (5) Many examples clearly demonstrate that resistance modules are interchangeable between plasmids belonging to different plasmid groups. This confirms that naturally occurring IncP-1 plasmids can act as shuttle vectors for several resistance determinants among a wide range of bacteria (Thomas, 2000b). In other words, a common pool of resistance determinants is shared by many different bacterial species as part of the horizontal gene pool. (6) Integrons on Tn402-like transposons were frequently found on IncP-1 plasmids from WWTP bacteria. Since these elements play an important role in the acquisition and exchange of genes encoding resistance to antibiotics and QACs, their presence on conjugative, broad-host-range plasmids is worrying. Indeed, linkage of genes encoding both antibiotic and QAC resistance allows for selection of drug resistance determinants by disinfectants. Moreover, the integron platform seems to be a target for the integration of additional nonintegron-specific resistance modules. (7) Plasmid-borne tripartite multidrug efflux systems broaden plasmid-mediated resistance spectra. Since many known MDR efflux systems also transport nonantibiotic compounds, these systems might be beneficial for the host bacterium even in habitats that are free from any antimicrobial drugs. (8) Release of bacteria carrying IncP-1 resistance plasmids with the final effluents of the WWTP into the environment promotes dissemination and recombination of resistance determinants among environmental bacteria. Thus the increasing emergence of pathogens possessing new resistance properties is not inconceivable.

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