Quantification of IncP-1 plasmid prevalence in environmental samples

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Abstract

To study the role of broad host range IncP-1 plasmids in bacterial adaptability to irregular environmental challenges, a quantitative real-time PCR was developed that specifically detects the conserved gene korB of all IncP-1 plasmids in environmental samples. IncP-1 plasmid dynamics in a biopurification system for pesticide wastes were analyzed.

Horizontal gene transfer by broad-host-range plasmids plays a vital role in the adaptation and robustness of bacteria to irregular or novel environmental challenges or opportunities (1, 2). Broad-host-range plasmids are also significantly contributing to the spread of antibiotic resistance (3). Plasmids of the incompatibility group IncP-1 (also called IncP in the classification scheme of Enterobacteriaceae plasmids) are assumed to especially foster the horizontal gene transfer because of their stable replication in a wide range of Gram-negative bacteria and their efficient conjugative transfer to an even wider range of taxa (4, 5). They consist of a conserved backbone carrying genes for plasmid persistence and conjugative transfer, and typically regions with diverse accessory genes that vary between plasmids. Accessory genes often encode antibiotic or metal resistances or degradative pathways (6).

IncP-1 plasmids were first discovered in bacteria from clinical specimens (7, 8), and subsequently were found in many geographic regions and diverse environments including agricultural soil, salt marsh, manure, compost, sewage, water, and sediment (6, 9-13).

However, the environmental distribution of IncP-1 plasmids and the factors promoting their frequency in bacterial communities are not well explored. High abundance of these plasmids seemed to be related to environmental disturbances like pollution (10, 11, 14). The detection in microbial community DNA was first based on PCR-amplification in combination with Southern blot hybridization of fragments of the trfA gene (12), which codes for the replication
initiation protein. These primers were developed based on sequences of the subgroups IncP-1α and β. The discovery of IncP-1 plasmids with largely divergent backbone sequences (9, 15, 16) led to the development of new primer systems for detection of trfA of the IncP-1 subgroups α, β, γ, δ, and ε (17). However, also these primers do not target all known IncP-1 plasmid types such as the newly described ζ subgroup (18) or pKS208 (accession JQ432564) which is most similar to the IncP-1γ plasmid pQKH54 (9). The problem to design one primer system to detect all known IncP-1 plasmid backbones for all IncP-1 plasmids based on trfA is caused by its relatively high evolutionary rate (Fig. 1), which is probably a result of adaptation to interacting host proteins in diverse hosts (20). Thus, trfA does not provide enough conserved sites as primer targets and, moreover, as an intermediate target for a TaqMan probe to enable specific quantification of IncP-1 plasmids in environmental DNA by a real-time PCR 5’ nuclease assay (qPCR). We searched the IncP-1 backbones for such a conserved region. All common backbone genes of the IncP-1 subgroups were aligned and relative evolutionary rates determined (Fig. 1). Among the most conserved regions found within trbC, trbE, traG, traI, and korB, the latter target gene was most suited to design a qPCR system. The korB product is evolutionarily constrained because it interacts with itself to form dimers, with IncC, KorA and multiple DNA binding sites of the plasmid as essential component of the partition system and the regulatory network of the plasmid (21, 22).

Two forward and three reverse primers were combined to minimize both degeneracy and mismatches to targets (Table 1). A single TaqMan probe was sufficient for qPCR with tested plasmids of all IncP-1 subgroups. An exception was the IncP-1γ plasmid pKS208, for which two mismatches to the first five 5’ bases of the TaqMan probe impeded 5’ nuclease activity. This problem could be solved by adding the TaqMan probe Pgζ to the PCR to target IncP-1γ and ζ plasmids including also more divergent variants (Table 1). Target DNA was amplified in reactions containing 5 µl DNA solution, 1.25 U TrueStart Taq DNA polymerase
and supplied buffer (Fermentas, St. Leon-Rot, Germany) in 50 µl, 0.2 mM of each deoxynucleoside triphosphate, 3.5 mM MgCl₂, 0.1 mg/ml bovine serum albumin (Fermentas), 0.4 µM of primers F and R, 0.2 µM of primers Fz, Rge and Rd, 0.3 µM of TaqMan probes P and Pg. Reactions were run at 5 min 95°C, and 40 cycles of 15 s 95°C, 15 s 54°C, 60 s 60°C in a real-time PCR system (CFX96, Bio-Rad, Munich, Germany). The annealing step at 54°C reduced the variance of cycle threshold values between cloned korB variants from six IncP-1 subgroups (data not shown), indicating an effect of sequence variation on amplification. To estimate the bias caused by this effect, the qPCR efficiencies of cloned korB fragments of plasmids representing the different subgroups and pKS208 were compared (Fig. 2). The amplification efficiencies of the korB gene variants did not differ significantly, as calculated from slopes of linear regression curves and test on parallelism (p=0.07, PROC GLM, statistical package SAS 9.3). Efficiency of the PCR was on average 88%, and detection limit was 10 copies. The lowest cycle thresholds with given initial concentrations were achieved with korB of pB10 and the highest with pKS208 and pKJK5. This variation between plasmids resulted in standard deviations of 0.4-0.5 log units at environmentally relevant concentrations (Fig. 3). The standard deviation increased with decreasing initial concentration. Around one third of this error could be explained by variation attributable to plasmid extraction and serial dilution of quantification standards, as shown by independent replicate preparations (Fig. 3).

The korB assay was applied to analyze the abundance and dynamics of IncP-1 plasmids in a large pesticide-degrading biofilter operated on a farm near Kortrijk, Belgium, which was previously characterized (23). Four spatially separated plots in the biofilter were sampled three times over a season with continuing applications of pesticide wastes, before start-up (March), during processing (July), and after close down (September). The biofilter, composed of coco chips, straw and soil, received 37 different active compounds including 16 halogenated aromatics (23). Total DNA was extracted from 0.5 g of 2 mm-sieved biofilter
samples using the FastPrep FP120 bead beating system for cell lysis and the FastDNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA) and purified using the GENECLEAN Spin Kit (MP Biomedicals). The copy numbers of korB were determined as described above, and 16S rRNA genes were quantified using the qPCR system developed by Suzuki et al. (24). In addition, trfA copies of IncP-1ε plasmids were measured, as previously described (10), to test whether more plasmids are detected by the qPCR targeting korB. A dilution series of plasmid pKJK5 was used as a common quantification standard for both korB and trfA. Gene copies were related to 16S rRNA gene copies to account for differences in bacteria concentration or amplification efficiency in the samples. The korB assay detected 2 to 4 times more IncP-1 plasmids than the trfA IncP-1ε assay, which was significant for all three samplings and which confirmed the intended broader specificity (Fig. 4). Concomitant with continued pesticide applications, the relative abundance of IncP-1 plasmids in the bacterial biofilter community increased from March till September reaching values of up to 0.2%. This might indicate an important contribution of IncP-1 plasmids to pesticide degradation, as previously suggested based on degradative pathways located on several IncP-1 plasmids (6).

Thus, our results indicate that we have successfully developed a rapid and sensitive method which allows the quantification of all known IncP-1 subgroups in environmental samples despite their diverse backbones. Its application will give further insight into the relative abundance of IncP-1 plasmids in bacterial populations, and their role in the spread of resistance genes, degradation pathways of recalcitrant compounds or other traits of the horizontal gene pool which allow a fast response of bacterial sub-populations to irregular environmental changes.
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References


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<th>Plasmid (accession)</th>
<th>Sg</th>
<th>Forward primers&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Reverse primers</th>
<th>TaqMan probes (5’-FAM, 3’-TAMRA)</th>
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<sup>a</sup> Sg: IncP-1 subgroup; <sup>b</sup> Mismatches of primers or TaqMan probes to target are underlined.
**Figure Legends**

**FIG. 1.** Relative evolutionary rates of genes common to the backbones of all known IncP-1 subgroups (α, β1, β2, γ, δ, ε, ζ). Genes of two representative plasmids of each subgroup (see Table 1) were aligned and position-by-position evolutionary rates determined using Mega5 (19). The scaled rates (average evolutionary rate across all sites is 1) are displayed as moving averages with a window width of 100 bp.

**FIG. 2.** Cycle threshold values related to initial gene copy numbers of cloned korB genes from six plasmids (pB10, pEST4011, pKJK5, pMCFB1, pQKH54, RP4) representing the different IncP-1 subgroups and pKS208. Solutions of korB fragments ligated into pGEM-T (Promega) with similar concentration (OD$_{260}$ 0.5, OD$_{260}$/OD$_{280}$ > 1.8) were serially diluted and applied to the developed real-time PCR 5’ nuclease assay. Grey area and dashed lines indicate 95% confidence limits and prediction limits of linear regression analysis, respectively.

**FIG. 3.** Standard deviations of korB gene quantification in dependence of the initial concentration, calculated from regression curves, for seven korB variants representing the IncP-1 subgroups and pKS208 (solid line), and for four independent preparations of the quantification standards for plasmids pEST4011 and pKJK5, respectively.

**FIG. 4.** Abundance of korB of IncP-1 plasmids and trfA of IncP-1ε plasmids in a biofilter that degraded various pesticides during the growing season. Plasmid copies were related to bacterial 16S rRNA gene copies (rrn) quantified from the same sample. Different letters indicate significant differences (Tukey test; spatially separated compartments within the 20 m x 1.2 m biofilter were used as replicates: n=3 for March, n=4 for July and September).