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Monitoring the Dissemination of the Broad-Host-Range Plasmid pB10 in Sediment Microcosms by Quantitative PCR▽†

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Studying the transfer of specific mobile genetic elements in complex environmental matrices remains difficult because suitable molecular tools are not yet available to back up classical culture-dependent approaches. In this report, we show that quantitative PCR could be used to monitor the dissemination of the broad-host-range plasmid pB10 in sediment microcosms. This approach lies in the differential measurement of the host and plasmid DNAs used to inoculate the microcosms, using a particular design of quantitative PCR primers/probes where we took advantage of the mosaic aspect of the bacterial genomes to achieve a highly specific quantitative PCR detection system.

Despite the progress made in our understanding of the basic mechanisms involved in horizontal gene transfer (12) and our awareness of their implications in bacterial evolution (1, 5), the dynamics of the genetic phenomenon involved still need to be evaluated in environmental contexts. As a matter of fact, our current vision of gene transfer is limited to either transfer experiments carried out *in vitro*, when talking about the basic mechanisms involved, or retrospective analyses, when studying the spread of relevant genetic markers among environmental bacteria. *In situ* transfer experiments were also of great interest, but they reached some limitations since no convenient tools could avoid selection and detection difficulties (11, 15, 16). Classically, the quantification of gene transfer involves culture-based methods and selection, which present major drawbacks when dealing with environmental samples (11, 15): first, it is believed that less than 1% of the environmental bacteria are cultivable, and second, the transferred genes may present a narrow host range of expression, which restricts their proper selection. As a consequence, culture-based approaches often lead to an underestimation of the bacterial counts, therefore limiting our perception of the extent of gene transfer in complex environments (11). Lately, elegant alternatives making use of plasmids tagged with green fluorescent protein genes for the fluorescent detection of transconjugants *in situ* have been developed (4, 11). Despite a better evaluation of the transfer frequencies, these approaches are restricted to the detection of transconjugants expressing fluorescent proteins well, and they still require the genetic alteration of the element studied. Molecular techniques such as PCR and quantitative PCR (qPCR) also offer the advantage of being both culture independent and gene expression independent since they are based solely on the specific detection of a given nucleotide sequence (10). Nevertheless, because many DNA markers are shared by various genetic entities/genomes, molecular approaches have mostly been restricted to the quantification of redundant conserved sequences in microbial communities rather than being used to specifically monitor the fate of a given mobile genetic element in complex environmental matrices. In this report, we show that the dissemination of the broad-host-range plasmid pB10 can be monitored in sediment microcosms by using a qPCR-based approach if specific primers are carefully designed.

Designing qPCR primers specific to pB10 and DH5α DNA. Monitoring of the fate of plasmid pB10 by qPCR following the inoculation of sediment microcosms required that both plasmid and donor bacteria (here *Escherichia coli* DH5α) could be quantified using highly specific primer/probe sets. Sequence comparisons of the plasmid with "nr" nucleotide databases using blastn software (NCBI) quickly turned off the possibility of targeting a specific pB10 marker, since extensive similarities with other genetic entities could be observed all along the pB10 sequence (not shown). Nevertheless, recent advances in genomics have taught us that bacterial genomes display a mosaic structure (6), where their originality lies in the particular combination of DNA blocks rather than in specific DNA sequences themselves. Taking advantage of this mosaic aspect, we used the "Primer Express v2.0" software program (Applied Biosystems) to elaborate a set of qPCR primers designed to prime on both sides of a unique junction between building blocks. The block organization of plasmid pB10 is illustrated in Fig. 1A. Basically, it consists of an IncPβ backbone having acquired a class 1 integron and four transposable elements, which exhibit various degrees of alteration (9). The junctions between pB10's blocks could easily be identified by sequence comparison, since each side of the junction matches sequences from different mobile genetic elements. Seven of these junctions were found in the transposons' region of pB10, and one of them, junction J3, was targeted for qPCR primer/probe design because of its putative stability (Fig. 1B and Table 1). First, junction J3 connects truncated versions of the trans-

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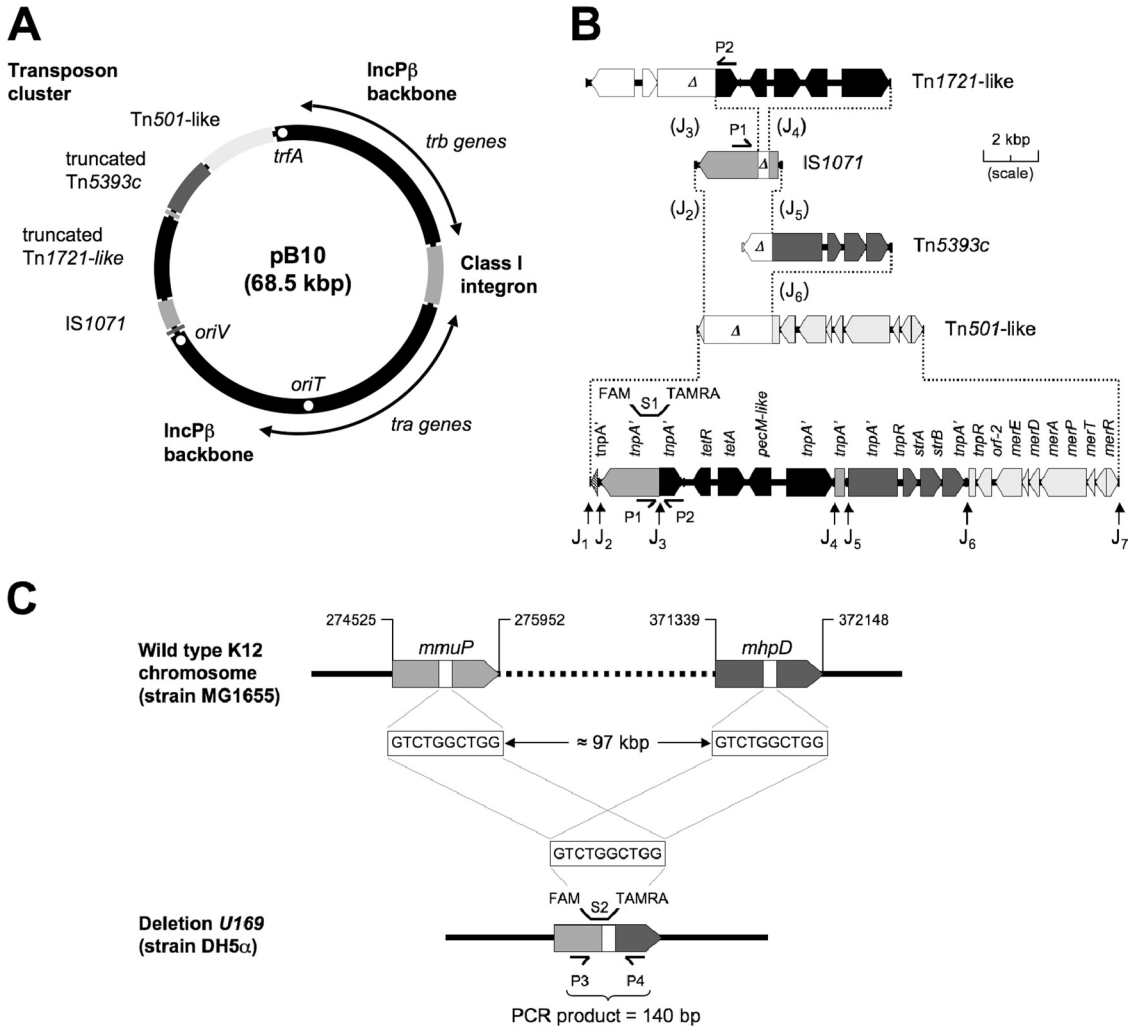

FIG. 1. Primer and probe design for the specific detection of pB10 and DH5α. (A) Schematic representation of the basic building blocks shaping plasmid pB10. Plasmid pB10 consists of a classical IncPβ backbone structure with the addition of a class I integron between the *trb* and *tra* gene clusters and the accumulation of four incomplete transposons between the *oriV* and the *trfA* genes. (B) Detailed genetic organization of the transposon cluster (bottom). The actual transposon cluster results from the successive insertion of four elements (Tn*501*-like, Tn*5383c*, IS*1071*, and Tn*1721*-like transposons) and the loss of genetic material (indicated by white boxes). Careful sequence comparison with known unaltered archetype transposons allows the identification of several original junctions (J) between each of these building blocks. Primers used for qPCR quantification of pB10 (P1 and P2) anneal on both sides of junction J3, while the S1 TaqMan probe anneals upstream of J3 (Table 1). (C) Primer design for the detection of DH5α by qPCR. The 97,239-bp *U169* deletion of DH5α results from the homologous recombination between two direct repeats of 10 bp (GTCTGGCTGG), thus joining distant sequences from the *mmuP* and *mhpD* genes. Primers and probes used for qPCR quantification of DH5α (P3/P4 and S2; Table 1) anneal on both sides of the remaining repeat.

posons Tn*1721*-like and IS*1071*, lacking either or both parts of the transposase genes and the recognized extremities, which makes them unable to transpose. Second, sequence analysis (see Methods in the supplemental material) of the pB10 transposons' region identified only poorly conserved repeats, making homologous recombination unlikely, apart from the two direct sequence repetitions covering the two *tnpA′* genes of the truncated Tn*1721*-like transposon next to the J3 junction (99.9% identity over 902 nucleotides). A recombination happening between these two repeats should delete the region in between but would leave an intact copy of the repeat, keeping junction J3 unaltered and still quantifiable by qPCR.

The design of qPCR primers specific to the genome of *E. coli* DH5α was also based on the identification of unique junctions between building blocks. Compared to the wild-type *E. coli* K12 chromosome, DH5α displays a few distinctive features, among which is the (*argF-lac*)*U169* deletion that is widely shared among K12 laboratory derivatives. The (*argF-lac*)*U169* deletion, sequenced in *E. coli* MC4100, corresponds to the loss of a 97,239-bp DNA fragment that brought together the distant sequences of the *mmuP* and *mhpD* genes (7). This distinctive feature was used to elaborate two primers designed to prime on both sides of the *U169* deletion (Fig. 1C and Table 1).

Testing specificities and sensitivities of primers in environmental samples. The specificity of each primer set and of their accompanying TaqMan probes (Table 1) was evaluated with a series of qPCR tests using template DNA extracted from 20

TABLE 1. Primers and probes used for specific detection of plasmid pB10 and *E. coli* DH5α by qPCR

Primer or probe	Sequence	Target	Priming coordinates[a]	Amplicon size, bp (purpose)
Primers				
P1	5′-CAATACCGAAGAAAGCATGCG-3′	*tnpA* of IS*1071* in pB10	45968–45988 (NC_004840)	135 (detection of pB10)
P2	5′-AGATATGGGTATAGAACAGCCGTCC-3′	*tnpA* of Tn*1721* in pB10	46078–46102 (NC_004840)	
P3	5′-ACCGGGTACATCATTTCC-3′	*mmuP* of K12	274654–274671 (U00096)	140 (detection of DH5α)
P4	5′-GCCCCGGTAAGAATGAT-3′	*mhpD* of K12	372917–372033 (U00096)	
Probes				
S1	(FAM)5′-CCTCCACGGTGCGCGCTG-3′ (TAMRA)	*tnpA* of IS*1071* in pB10	45990–46007 (NC_004840)	(detection of pB10)
S2	(FAM)5′-TCTGATTGGTGCGCTGGTGGTCT GG-3′(TAMRA)	*U169* deletion	274704–274729 (U00096)	(detection of DH5α)

[a] Accession numbers of the template DNA sequences are indicated in parentheses.

different environmental samples originating from different locations and/or collected at different times (see Table S1 in the supplemental material). Total environmental DNA was extracted using a method adapted from the work of Porteous et al. (8) (see the supplemental material for details), and qPCR assays were run with and without the addition of DNA from DH5α or pB10. As expected, no amplification signal could be detected unless DNA from pB10 or DH5α was added to the qPCR reactions, thus demonstrating the uniqueness of each of the block junctions targeted. The sensitivity of the qPCR design to quantify pB10 and its host from complex environmental matrices was determined by inoculating environmental samples with known quantities of DH5α/pB10 bacteria, followed by immediate extraction of total DNA and quantification by qPCR. Considering an average of 2 pB10 copies per cell (typically 1 to 3 per cell for IncP plasmids), it appeared that only *ca.* 20% of the expected pB10 and 0.25% of the expected DH5α DNA could be recovered and quantified from sediment samples, accounting for an overestimated pB10/DH5α ratio of *ca.* 150. This discrepancy probably reflects different efficiencies of recovering plasmid DNA as opposed to long and fragile chromosomal DNA. This also means that quantitative data have to be analyzed in terms of rate of disappearance/appearance for a given target DNA rather than in terms of absolute quantities.

Monitoring the fate of pB10 sediment microcosms. The pertinence of the primers/probes designed was demonstrated in an experiment aiming at monitoring the fate of pB10 and the donor bacteria in river sediment microcosms by qPCR. A set of 800 ml microcosms, consisting of a blended mixture of river water and sediments sampled in the Moselle River (Lorraine, France), were inoculated with 2.10^8 DH5α/pB10 bacteria (9) and were maintained at 20°C for 5 days with gentle aeration and regular nutritive amendment (see the supplemental material for details). Microcosms' sediments were sampled at intervals, and total community DNA was extracted as previously described before quantification of both pB10 and DH5α DNA by qPCR (see the supplemental material for details). As shown in Fig. 2A, the concentration of pB10 detected remained relatively stable over the duration of the microcosm operation (5 days). Interestingly, the fate of DH5α differs drastically from that of pB10 (Fig. 2B), with a complete loss of the DH5α DNA

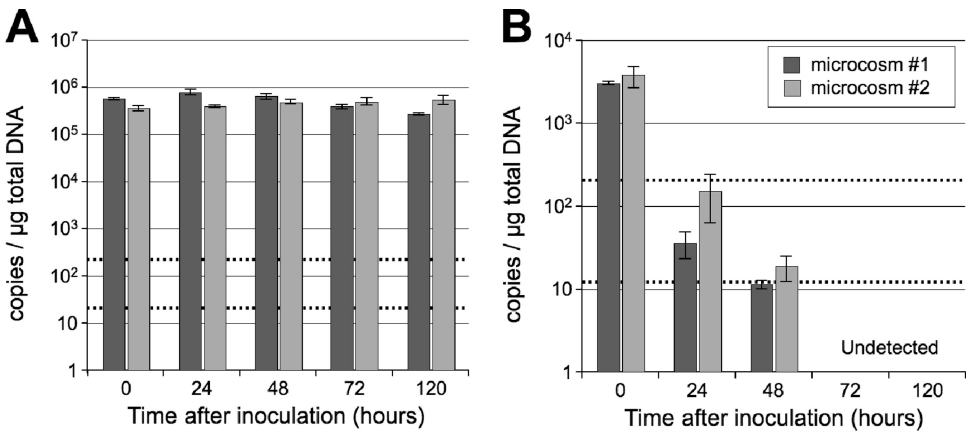

FIG. 2. Monitoring the fate of pB10 (A) and DH5α (B) by qPCR over a 5-day period. Two microcosms were set up with an identical sediment sample and run independently. The same community DNA was used for the quantification of both pB10 and DH5α by qPCR. Each qPCR was carried out in triplicate (error bars show standard deviations). All data were normalized to total community DNA and expressed as a number of copies per μg of community DNA. Top dotted lines represent limits below which the linearity between the C_T values and log DNA copy number is lost (see Methods in the supplemental material for details). Bottom dotted lines correspond to a detection limit of 1 DNA copy per reaction. In some instances, values slightly below 1 DNA copy per reaction resulted from the mathematical conversion of C_T values too close to the detection limit.

detected within 48 h, indicating that the apparent stability of pB10 is likely to be due to an early invasion of the bacterial community. Control microcosms with uninoculated sediments were systematically run, sampled, and treated in parallel. For these microcosms, undetected by qPCR DH5α and pB10 remained throughout the experiment, thus showing that the pB10 detected in the inoculated microcosms did not correspond to the unexpected resurgence of a closely related sequence originating from the sediment population. Additionally, a second set of controlled microcosms inoculated with naked pB10 DNA showed that the amount of plasmid decreased steadily from time zero and became undetectable after 48 h (not shown). This rapid loss (2 logs in 48 h) excludes the possibility that pB10 could have persisted extracellularly in the experiment depicted in Fig. 2.

Discussion. To the best of our knowledge, this is the first time that the dissemination of a broad-host-range plasmid has been monitored at the molecular level in a complex environment. This was made possible by focusing the design of qPCR primers around unique genome structures—building block junctions—rather than looking for specific DNA markers that are rather rare in complex communities, especially when promiscuous IncP plasmids such as pB10 are involved. Because this design is specific enough to detect and quantify a given DNA segment in a complex DNA mixture, it now opens up a wide range of possibilities in matters of environmental genetics for studying the dynamics of gene transfer in complex environmental matrices without disturbing the indigenous communities with excessive donor inoculums, as can be the case with classical methods (see reference 14 and references therein).

Despite avoiding biases associated with the culture of microorganisms, the qPCR approach described here has some limitations when it comes to quantification. Indeed, the data generated in this study were treated as if DNA could be recovered with the same 100% efficiency whatever its nature (plasmid or genomic), its origin (from pure culture or microcosms), or the purification protocol used, which is not really the case. Actually, while generating highly accurate standard curves (threshold cycle [C_T] versus concentration of pure DNA), we could obtain an extinction of the signal for expected concentrations of template DNA close to 1 copy per qPCR assay (*ca.* 1 and 5 copies per qPCR assay for pB10 and DH5α, respectively), thus attesting that the concentrations of the targeted sequences were properly estimated. In fact, difficulties arose when extracting DNA from environmental samples, because pB10 and DH5α genomic DNA could not be recovered with the same level of efficiency, which implies that the two DNA templates are not directly comparable from a quantitative point of view. Nonetheless, with the concentration of the recovered DNA remaining proportional to the initial concentration in a given environmental sample, it is possible to monitor accurately the fate of a particular DNA sequence over time, as far as it involves identical extraction and quantification conditions. From this, rates of appearance/disappearance can be obtained independently for different DNA targets, and compared between each other, as has been done here for pB10 and DH5α DNA, since they do not relate absolute quantities of DNA but the evolution of these quantities.

In order to better highlight the transfer of pB10, as opposed to its stable maintenance in the donor strain, we deliberately chose a donor bacterium with poor chances of survival in sediment microcosms, first because this is not the natural habitat of *E. coli* and second because the numerous genetic alterations of the laboratory-adapted strain DH5α are likely to reduce even further its fitness in such an environment. In this respect, the data presented here evidenced that following the microcosm inoculation, the amount of DH5α DNA quickly decreased, becoming undetectable after 3 days. This is in good accordance with previous observations made by Top et al. (13) showing that *E. coli* survival in soil was deeply influenced by the availability of favorable substrates and the presence of indigenous competitors. In their work, Top et al. (13) also showed that the appearance of transconjugants correlated with the survival of the *E. coli* donor bacteria. Nevertheless, it should be remembered that in these experiments, the plate-counting approach used narrowed the study to the gene transmission toward one recipient strain only, while any indigenous recipient could be considered with the molecular approach we developed.

Interestingly enough, we observed that pB10 was maintained at a steady-state level while the *E. coli* donor was quickly disappearing, which tends to show that most of the transconjugants formed at an early stage in the microcosm experiment. This is reminiscent of previous observations made by Fulthorpe and Whyndham (3), for instance, where a sediment microcosm inoculated with the 3-chlorobenzoate (3Cba)-degrading strain *Alcaligenes* sp. BR60 kept a constant level of 3Cba degraders under selective pressure while BR60 disappeared. Further analyses also showed that the mobile genetic element hosting the catabolic genes of the donor strain BR60, a Tn*5271* catabolic transposon inserted into the broad-host-range plasmid pBRC60, was detected at a constant level throughout the experiment even after the loss of the donor. The hypothesis we are currently favoring to explain the apparent stability of the pB10 copy number in our microcosm experiment relates to the spatial structure of the habitat. Recently it has been shown that pB10 can invade a wider part of the recipient population if the spatial structure of the habitat is regularly disturbed (2). Indeed, in a complex environmental matrix, such as sediments, the populations are likely to organize in clusters physically isolated from one another and the transfer of pB10 would be limited to adjacent cells. Further transfer experiments with microcosms exposed to various shearing protocols should highlight the relative importance of the spatial structure compared to other environmental parameters (e.g., temperature, nutrients, origin of environmental samples, etc.).

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