Complete Nucleotide Sequence and Analysis of Two Conjugative Broad Host Range Plasmids from a Marine Microbial Biofilm

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Abstract

The complete nucleotide sequence of plasmids pMCBF1 and pMCBF6 was determined and analyzed. pMCBF1 and pMCBF6 form a novel clade within the IncP-1 plasmid family designated IncP-1 subclade. The plasmids were exogenously isolated earlier from a marine biofilm. pMCBF1 (62,689 base pairs; bp) and pMCBF6 (66,729 bp) have identical backbones, but differ in their mercury resistance transposons. pMCBF1 carries Tn5053 and pMCBF6 carries Tn5058. Both are flanked by 5 bp direct repeats, typical of replicative transposition. Both insertions are in the vicinity of a resolvase gene in the backbone, supporting the idea that both transposons are ‘‘res-site hunters’’ that preferably insert close to and use external resolvase functions. The similarity of the backbones indicates recent insertion of the two transposons and the ongoing dynamics of plasmid evolution in marine biofilms. Both plasmids also carry the insertion sequence IS611, albeit without flanking repeats. IS611 is located in an unusual site within the control region of the plasmid. In contrast to most known IncP-1 plasmids the pMCBF1/pMCBF6 backbone has no insert between the replication initiation gene (trfA) and the vegetative replication origin (oriV). One pMCBF1/pMCBF6 block of about 2.5 kilo bases (kb) has no similarity with known sequences in the databases. Furthermore, insertion of three genes with similarity to the multidrug efflux pump operon mexEF and a gene from the NodT family of the tripartite multi-drug resistance-nodulation-division (RND) system in Pseudomonas aeruginosa was found. They do not seem to confer antibiotic resistance to the hosts of pMCBF1/pMCBF6, but the presence of RND on promiscuous plasmids may have serious implications for the spread of antibiotic multi-resistance.

Introduction

Plasmids are independent from the bacterial host chromosome and conjugative broad-host range (BHR) plasmids can transfer between many different species in a bacterial community. Thus, plasmids are a particularly fluid part of the bacterial genome and one that may provide the cell with new traits that allow adaptation to selection forces, especially to transient environmental changes and challenges. Plasmids are likely to evolve differently in different bacterial backgrounds [1] and might also be influenced by environmental conditions [2]. Thus analysis of plasmids from different environments is important for our understanding of these important mobile elements (MGE). There are a number of studies about plasmids in marine environments [3,4,5,6,7,8,9,10,11], as well as reports about the role of marine plasmids in antibiotic resistance [12,13,14,15] and population dynamics [16,17], for excellent reviews on this topic see [18,19]. Still, compared with other BHR plasmids isolated from marine environments, it is interesting to analyze both their backbone genes and their accessory genes, and to compare these with other IncP-1 plasmids. Here we present a detailed analysis of pMCBF1 and pMCBF6, a biofilm, was shown to have a BHR and transferred to many Gram-negative bacteria, including Planctomyces maris [21]. We also measured comparatively high transfer rates of pMCBF1 from a P. putida donor to indigenous bacteria, directly in seawater [4]. pMCBF1 was therefore an interesting BHR marine biofilm plasmid to characterize further. We determined the complete nucleotide sequences of pMCBF1 and the related pMCBF6, and a brief summary of the plasmids was presented earlier [1]. It was also shown that they form a novel clade within the promiscuous IncP-1 plasmid incompatibility group [1]. Since pMCBF1 and pMCBF6 are the only representatives of their IncP-1 clade, and except for pMLUA1, pMLU3 and pMUA4 [22], so far the only sequenced IncP-1 plasmids isolated from marine environments, it is interesting to analyze both their backbone genes and their accessory genes, and to compare these with other IncP-1 plasmids. Here we present a detailed analysis of pMCBF1 and pMCBF6.

Materials and Methods

Bacterial strains, plasmids and growth conditions

P. putida UWC1 [23] and P. putida KT2440 [24] with pMCBF1 and pMCBF6 were grown overnight at 26°C in Luria-Bertani
media [25] with 10 g NaCl per liter and supplemented with HgCl₂ at 17 mg per liter. Escherichia coli with pMCBF1 and pMCBF6 were grown overnight at 37°C in the same medium supplemented with ampicillin (50 mg/liter).

Mercury and antibiotic resistance tests

To test for phenyl mercury resistance, P. putida KT2440 with either pMCBF1 or pMCBF6 were grown overnight and spread as lawns on LB plates. Discs that were dipped in solutions of phenyl mercury chloride (saturated solution in ether) were dried and placed on the lawns. Plates were inoculated overnight at 30°C after which the diameters of clearing zones around the discs were recorded. P. putida KT2440 without plasmids was used as controls. For the antibiotic resistance tests susceptibility discs (OXOID, UK) were placed on bacterial lawns of E. coli HB101 [26] and CAG [27] with and without pMCBF1 and pMCBF6 and incubated in 37°C overnight and clearing zones recorded as above.

Sequencing and sequence analysis

Sequencing of pMCBF1 and pMCBF6 was performed earlier in our laboratory using standard techniques [1]. Sequence alignments presented here were created using the eBioX program [29], and the similarity analysis was carried out using the SimPlot program [29]. The content of the genomes of the IncP-1 plasmids vary and all genes are not present in all plasmids [1]. The network constructed here was based on the largest segment that we could identify in which all genes were present in all analyzed plasmids (TraC to TraM).

Nucleotide sequence accession numbers

The complete sequences from pMCBF1 and pMCBF6 are deposited with GeneBank CoreNucleotide (accession #AY950444 and EF107516, respectively).

Results and Discussion

pMCBF1 and pMCBF6 represent the first fully sequenced IncP-1 plasmids from marine environments. Generally the difference between the IncP-1 plasmids sequenced so far lies in the various insertions of resistance and catabolic genes, often as part of MGEs, onto a more or less conserved backbone structure. pMCBF1 and pMCBF6 are obvious examples of this since they differ only in accessory genes and functions such as transposons, various resistance functions, IS elements and multi-drug efflux systems. There are various genetic distances between the pMCBF1/pMCBF6 backbone genes and the corresponding genes in plasmids from the other clades, which is probably explained by the history of recombination [1]. As an example, most of the backbone genes such as replication, conjugation and stable maintenance genes share 58–95% similarity with genes from plasmids pADP-1 [40], pB4 [41], R751 [31] and pTSA [42] from the β clade, which is the most studied IncP-1 clade.

The origin of replication (oriV) of pMCBF1/pMCBF6 resembles other IncP-1 plasmids. The nine TrfA DNA binding sites (iterons) in the vicinity of the pMCBF1/pMCBF6 oriV have the 17 bp consensus sequence N(T/C/G)GCCCTC(T/A/T/G)CT(A/T)G/TCA, and have been conserved to some degree compared to both RK2 and R751. The R751 iterons by comparison have the sequence (A/C/G)NGCCC(T/C)A(A/T)(A/G/C)T(A/G)T(C/T)A, and have been conserved to some degree compared to both RK2 and R751. The R751 iterons by comparison have the sequence (A/C/G)NGCCC(T/C)A(A/T)(A/G/C)T(A/G)T(C/T)A, and have been conserved to some degree compared to both RK2 and R751.

The TrfA protein is necessary for the replication of iteron containing theta replication plasmids (e.g. [43]) and is thought to be used as a base for plasmid classification [44]. We suggested earlier that pMCBF1 and pMCBF6 had replication functions that were different from known plasmid incompatibility groups based on lack of hybridization with the inc/rep Couturier probes [20]. However, the general lack of similarity of the Couturier plasmids with many environmentally isolated plasmids has been noted in several investigations [9,18,45,46,47]. We now know that the IncP-1 Couturier probe only detects the IncP-1 α clade [48]. In fact, the nucleotide similarity between pMCBF1/pMCBF6 and the Couturier IncP-1 probe is only about 60% and these plasmids could therefore not be targeted by the probe.

The pMCBF1/pMCBF6 backbone has no insert between the replication initiation gene trfA and the oriV. Except for the IncP-1 β2 plasmids pBP136 and pA1, most other IncP-1 plasmids have an insert, usually several kilobases (kb), that separates trfA and oriV.

Just as for the IncP-1 β plasmids, pMCBF1/pMCBF6 lack the parABCD locus that mediates accurate plasmid segregation. Surface exclusion systems also protect the resident plasmid from competition, by preventing new plasmids to enter the cell. pMCBF1/pMCBF6 carry the traK homologue, which confers surface exclusion in plasmid RK2. We know from recombination analyses that pMCBF1/pMCBF6 have been involved in recombination events [1], which strongly suggest that these plasmids have been in the
same host cell as other IncP-1 plasmids [1]. Thus, it seems that the surface exclusion systems of pMCBF1/pMCBF6 have a “leakiness” that allow recombination to occur.

One block of about 2.5 kb, between the inserted HgR transposons and the trb section (marked with a question mark in Fig.1) has a low similarity (73% identity) to *Methylophaga* sp. JAM7 [49].

Accessory genes, transposons and insertion sequences

The DNA segment bearing the mercury resistance determinant on pMCBF1 (Fig. 1B) shows 99% nucleotide similarity to Tn5053 [50]. There are five base pair direct repeats in the flanking plasmid DNA, which are typically formed after Tn5053 transposition [50]. Interestingly, Tn5053 in pMCBF1 is inserted close to the resolvase gene (*resA*) in the backbone (Fig. 1A) which supports the suggestion that Tn5053 is a “resolvase-site-hunter” that insert in “hot-spots”
sequence similarities suggesting that these transposons are to several Tn15,000–40,000 years in permafrost grounds [56]. When compared to other IncP-1 plasmids such as pIJB1, pWEC911 and pTP6, events involving these elements [56]. Tn that Tn1 is part of the Tn superfamily of diverse elements including retroviruses, phage Mu, inverted repeats (IR) that form the ends of Tn5053, inserted by some other recombination mechanism than replicative transposition, the insertion point of IS501, 5041D, 5718 and others, it was suggested that Tn5058 was formed by a complicated series of recombination events involving these elements [56]. Tn5058 is also carried by other Inc-P-1 plasmids such as pJBJ1, pWEC911 and pTP6. Interestingly, Tn5058 has been detected in bacteria preserved for 15,000–40,000 years in permafrost grounds [56]. When compared to several Tn5058 collected in modern days, there were high sequence similarities suggesting that these transposons are genetically stable over time. We compared the Tn5058 in plasmid pMCBF6 with the Tn5058 isolated from permafrost, using the sliding window protocol implemented in the SimPlot program. Our results support previous suggestions that the Tn5058 is indeed well conserved and there is a high nucleotide identity between the transposon Tn5058 isolated here and the transposon Tn5058 isolated from permafrost in most regions. Least conserved was the gene traC, with a 200 bp region of only 80% identity and merR and mer with regions of less than 95% identity between the Tn5058 in pMCBF6 and the Tn5058 isolated from permafrost. Tn5058 has two copies of merR, merB and merD. Interestingly, it was suggested that the duplications in some of the mer genes in Tn5041D were the result of an integration, via homologous recombination, of a mer containing circular DNA structure [55]. The circular cassette was speculated to originate from an ancestral donor in which the mer genes were flanked by IS elements. The duplication of mer genes in Tn5058 might also have been the result of a similar event [55]. Just as for Tn5053 in pMCBF1, Tn5058 is flanked by 5 base pair direct repeats in pMCBF6, which strongly indicate a transposition event [50,52]. These flanking direct repeats are also seen when Tn5058 is inserted in IncP-1 plasmid pJBJ1. The insertion of Tn5058 in pMCBF6 is only 63 bp from the site where Tn5053 inserted in pMCBF1, which is close to the resolvase gene (rec4) in the plasmid backbone. The insertion site further confirms that Tn5058 is a “res-site hunter” [51].

The sequence data suggest that Tn5053 confers mercury resistance towards inorganic mercury while Tn5058 also carries resistance towards organo-mercury compounds. When the two plasmids were tested for phenyl mercury chloride (PhHg) resistance the diameter of the clearing zones around PhHg discs was only 6 mm for P. putida 2440(pMCBF6) but 13 mm for P. putida 2440(pMCBF1) and the plasmid-free P. putida 2440, confirming the PhHg resistance of pMCBF6 but not of pMCBF1. A 2982 bp sequence between kflA and merE in both pMCBF1 and pMCBF6 shows 99% (nucleotide) similarity with insertion sequence ISP1 (Fig. 1A). The 24 bp IR regions with 4 mismatches characteristic of ISP1 [57] are found in pMCBF6/pMCBF1. ISP1 is related to the ISL3 family, but is larger than the usual ISL3 elements which are 1300–1500 bp. ISP1 has been found in several copies in Pseudomonas stutzeri, where it has inserted into, and inactivated, catabolic genes [57], and in Versinia ruckeri plasmid pYR1 [58]. Interestingly, ISP1 was also recently found on plasmid pAMEC615 from the marine Alteromonas macleodii isolated from the English Channel [59]. In Pseudomonas stutzeri, ISP1 was flanked by eight bp direct repeats (DR), indicating a replicative transposition event [57], but no such flanking DR was found in any of pMCBF1/pMCBF6, pAMEC615 or pYR1. If DR were formed during a replicative transposition in these plasmids, they may have degenerated with time, perhaps because these sequences are not under selective pressure. Alternatively, the ISP1 was inserted by some other recombination mechanism than replicative transposition. The insertion point of ISP1 in pMCBF1/6, within the plasmid control region between kflA and upf45,8, seems to be unique among IncP-1 plasmids, indicating that insertion points other than the ones between tra and teb regions and between the replication region and oriV, are possible.

A region containing a putative remnant of an insertion event is located between klcA and klcB. This region contains an ORF with closest similarity (74%) to a putative transposable, as well as an IstB homolog. IstB is associated with IS21 family insertion sequences. The function of IstB is unknown, but it may assist in transposition [60]. We find no DR or IR as signs of a recent insertion. Insertions of transposons in the klcA/klcB region are seen also in IncP-1 plasmid RP1/RK2 [61].

Figure 2. Phylogenetic network of clades of the IncP-1 plasmid family. The network is based on representative plasmids from each of the twelve previously described phylogenetic clades of the IncP-1 plasmid family. The network is based on the genetic segment harboring the traC - traM genes for all analyzed plasmids. The two plasmids pMCBF1 and pMCBF6 described in this study are highlighted in bold. Clades without designated names are marked with *. Previously described intra-clade recombinants [1] were not included in the analysis. The figure is updated from [1]. doi:10.1371/journal.pone.0092321.g002
Adjacent to ISP/P is an inserted region that shows a high similarity to part of the recently sequenced plasmid pALDIE201 from *Alcyconiphilus denitrificans* isolated from anaerobic sewer sludge [62]. Furthermore, three genes within this region show similarity (73-87%) to mexEF and a gene from the NodT family, which are part of a partite multi-drug resistance-nodulation-division (RND) families. Such efflux systems are common on chromosomes of many Gram-negative bacteria [63], e.g. the *mexEF-oprN* in *Pseudomonas aeruginosa*. The Inc-P-1 β plasmid pB4 has a region with about 80% similarity to a RND efflux system in *P. denitrificans*, this was the first plasmid that was shown to carry a RND efflux system [41]. Adjacent to the RND region in pB4 a putative transposon terminus was found close to a Tn3-like transposase, and the authors speculated that the RND efflux system might have been transferred to pB4 from the chromosome of an unknown gram-negative bacterium by transpositional cointegrate formation [41]. Although ISP/P is found adjacent to the mex-region in pMCBF6/pMCBF1, we do not know if this IS element was involved in the mex insertion. The MexEF-OprN system in *P. aeruginosa* confers resistance to aromatic hydrocarbons, fluoroquinolones, chloramphenicol, triclosan and trimethoprim [63]. Our experimental data shows that when tested in disk diffusion tests for antibiotic sensitivity on agar plates, *E. coli* with pMCBF1 or pMCBF6 did not confer a higher resistance to chloramphenicol, nalidixic acid or trimethoprim than plasmid free cells. Further studies are needed to define the possible function of MexEF-OprN in pMCBF1/pMCBF6. Our finding that plasmids from a marine biofilm carry a multi-drug efflux system shows that pB4 is not unique, and that many different efflux systems may potentially be borne by promiscuous plasmids. The possibly serious implications of multi-drug efflux systems on such plasmids and the effects this may have on the spread of multi-resistance to human pathogens has been discussed [41].

The majority of the well-known IncP-1 plasmids, and plasmids in general, originate from soil environments or from a clinical environment and other man-made systems, such as wastewater treatment plants [35]. pMCBF1/pMCBF6 are among the first IncP-1 plasmids that are sequenced from marine environments. Three Inc-P-1c plasmids (pMLUA1, pMLUA3, pMLUA4) were recently isolated from the air-water interface from an estuary in Portugal [22,64]. Analysis of these, and other plasmids from marine bacteria, has often revealed new features [7,10,11,22]. Analysis of the pMCBF1/pMCBF6 plasmids also showed, among other things, that they form a new clade within the Inc-P-1 plasmid group.

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Author Contributions

Conceived and designed the experiments: MB MH. Performed the experiments: MB. Analyzed the data: PN MB MH. Contributed reagents/materials/analysis tools: PN MB. Wrote the paper: PN MB MH.
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