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Identification and Sequence of the Basic Replication Region of a Broad-Host-Range Plasmid Isolated from *Thiobacillus ferrooxidans*

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The minimum region required for replication of the broad-host-range *Thiobacillus ferrooxidans* plasmid pTF-FC2 in *Escherichia coli* was shown to be contained on a 2.05-kilobase fragment of DNA. A 184-base-pair fragment that was required in *cis* for plasmid replication was identified. This region was also involved in plasmid incompatibility. Nucleotide sequencing of this region revealed three perfectly conserved 22-base-pair tandemly repeated sequences. A comparison of this region with the equivalent region of the broad-host-range plasmid R1162 showed that the repeated sequences had 60% nucleotide homology. The 106-base-pair region immediately adjacent to the repeated sequences was 75% homologous. These plasmids were compatible.

Plasmid pTF-FC2 is a cryptic, 12.4-kilobase (kb) plasmid that was isolated from the acidophilic autotroph *Thiobacillus ferrooxidans*. The plasmid was cloned into the vector pBR325 and shown to be capable of replication in *Escherichia coli* from an origin of replication located on the *T. ferrooxidans* plasmid (17). The plasmid was mobilized between *E. coli* strains by the IncP plasmid RP4 (20), and the region responsible for mobilization was located (18). Although the extent of the host range of the *T. ferrooxidans* plasmid has not been widely investigated, it has been shown to replicate in *Pseudomonas aeruginosa* (18), *Klebsiella pneumoniae* (unpublished results), and *Thiobacillus novellus* (19).

The molecular mechanism and control of plasmid replication have become the subject of extensive research in recent years (22). Of particular interest is the ability of certain plasmids to replicate in a large number of bacterial species, whereas the host range of other plasmids is restricted to one or a few closely related species. Relatively few small broad-host-range plasmids have been identified. The most extensively studied of these are the plasmids of the IncQ incompatibility group. These include the similar or identical plasmids RSF1010 (5), R1162 (12), and R300B (2). An investigation into the molecular mechanism of replication of pTF-FC2 may help to identify features that are associated with the broad-host-range character of these plasmids. In addition, since *T. ferrooxidans* is used to leach a variety of metals from their ores (4), these studies can contribute to the construction of cloning vectors for this industrially important organism.

We report the isolation of the basic replication region of the broad-host-range *T. ferrooxidans* plasmid pTF-FC2 in *E. coli*. A region required for replication in *cis* when the intact replicon is supplied in *trans* was identified. This region was sequenced and compared with those of the IncQ plasmids.

MATERIALS AND METHODS

Bacterial strains and plasmids. Plasmids were maintained in *E. coli* K-12 strains JM105 (23) and LK111 (24). The *E. coli* DNA polymerase-deficient (*polA1*) strain GW125a (gift from G. Walker, Massachusetts Institute of Technology, Cambridge) was used to test for *polA*-independent plasmid replication. Plasmids used in this study are listed in Table 1.

Media and general recombinant DNA techniques. Bacteria were grown in Luria broth (LB) and on Luria agar (LA) plates at 37°C. Unless stated otherwise, 100 μg of ampicillin per ml and 25 μg of chloramphenicol per ml were used for plasmid selection. Plasmid DNA was prepared by the method of Ish-Horowicz and Burke (7), and general DNA cloning techniques were as described in Maniatis et al. (11).

Determination of plasmid copy number. All plasmids tested for copy number were grown in the *polA E. coli* mutant GW125a. Two methods were used to determine plasmid copy number. In the first, total DNA was isolated from cells carrying the relevant plasmids. Precise quantities of total DNA were digested with *Pst*I restriction endonuclease, and the fragments were separated by electrophoresis in agarose gels. The gels were blotted and hybridized to nick-translated pDER412. The intensity of the hybridization signals was compared with that of signals obtained from known concentrations of plasmid. The quantity of plasmid relative to the *E. coli* chromosomal DNA was calculated by using the relative molecular sizes of the *E. coli* chromosome (4,000 kb) and pTV100 (8 kb). In this way it was possible to determine the number of plasmids per chromosome.

In the second method, plasmid copy number relative to pTV100 was determined by single-cell resistance to ampicillin by the method of Nördstrom et al. (14). Bacteria were grown overnight in LB containing concentrated ampicillin, and 10^5-, 10^6-, and 10^7-fold dilutions were spread onto LA plates containing various concentrations of ampicillin. Colonies were counted after incubation overnight, and the amount of ampicillin required to give 50% survival was determined.

Test for incompatibility. The ability of fragments of pTF-FC2 to displace pDER412 was used as the test for incompatibility. Plasmids to be tested were transformed into LK111 containing pDER412, and transformants were plated on LA containing ampicillin. Six colonies from each transformation were streaked onto separate plates containing ampicillin to give single colonies. From each plate, 10 colonies were picked and tested for resistance to chloramphenicol. Incompatibility with the IncQ plasmids was tested by substituting pKE462 for pDER412 and using tetracycline resistance for selection of pKE462.

Plasmid deletions and DNA sequencing. Deletions of

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TABLE 1. Plasmids used in this study

Plasmid	Antibiotic resistance	Description	Reference or source
pBR325	Tc^r Cm^r Apr^r		3
pUC19	Ap^r		23
Bluescript SK	Ap^r		Stratagene, San Diego, Calif.
pKE462	Tc^r As^r	7.5-kb *Eco*RI fragment from R46 carrying Tc^r and arsenic resistance cloned into R300B	Kim Ellis, Hammersmith, London
pDER401	Cm^r Tc^r	pTF-FC2 cloned into *Pst*I site of pBR325	16
pTT100	Ap	pTF-FC2 cloned into *Pst*I site of Bluescript SK	This study
pDER412	Cm	*Xho*I-*Sal*I deletion of pDER401 resulting in deletion of pBR325 origin of replication	16

pTV400 were made by the exonuclease III method of Henikoff (6). The DNA sequence was obtained from both strands by the chain-termination sequencing method with the Sequenase DNA sequencing kit of United States Biochemical Corp., Cleveland, Ohio.

RESULTS

***polA* independence of the pTF-FC2 origin of replication.** The *T. ferrooxidans* plasmid pTF-FC2 was previously cloned into the *Pst*I site of the vector pBR325, and the *oriV* of pBR325 was removed, to produce recombinant plasmids pDER401 and pDER412, respectively (17). We investigated whether the replication of the recombinant plasmids was independent of the *E. coli polA* gene. Plasmids pBR325, pDER401, and pDER412 were transformed into the *polA* mutant GW125a, and the transformants were tested for the presence of the plasmids by selection for growth on chloramphenicol. The presence of a plasmid was confirmed by restriction analysis of the plasmids isolated from transformants. Only pDER401 and pDER412, which contain pTF-FC2 plasmid DNA, were able to replicate in GW125a. Plasmids based on the pMB1/ColE origin such as pBR325 and related plasmids are dependent on *polA* for replication (9). Since the replication of the pTF-FC2 plasmid is *polA* independent, the ability to replicate in GW125a was used to identify fragments of pTF-FC2 involved in replication.

Determination of the minimal replicon of pTF-FC2 in *E. coli*. Plasmid pTF-FC2 was cloned into the Bluescript SK vector pTT100, and a number of subclones were constructed to identify the region of DNA involved in plasmid replication. A restriction map of pTT100 is shown in Fig. 1. The 5.0-kb *Cla*I-*Eco*RI fragment cloned into the Bluescript SK vector pTV100 was able to replicate in *E. coli* GW125a. Bluescript SK subclones pTV200 and pTV300, which contain the 2.6-kb *Cla*I-*Apa*I fragment and the 2.4-kb *Apa*I-*Eco*RI fragment, respectively, lost their *polA* independence. To identify the basic replicon further, a partial *Sau*3A library of pTV100 was constructed in the vector pUC19 and transformed into GW125a. A recombinant plasmid, pTV400, which contained a 3.2-kb pTF-FC fragment, was isolated. Exonuclease III shortening was used to produce a series of plasmids in which the 3.2-kb pTF-FC2 fragment had been progressively deleted from either end. Plasmids containing shortened pTV400 fragments were tested for the ability to replicate in GW125a, and the basic replication region was identified (Fig. 1). Plasmid pTV4101, which had a 600-base-pair (bp) deletion from the left-hand *Sau*3A site of pTV400, was able to replicate in GW125a, whereas pTV4111, which had an 850-bp deletion, had lost this ability. A 300-bp deletion from the right-hand *Sau*3A site of pTV400 (pTV4200) had no effect on replication, whereas a plasmid with a 650-bp deletion (pTV4220) was unable to replicate. *E. coli* GW125a cells containing pTV4210 with a 400-bp deletion produced colonies that were markedly smaller than those of cells carrying either pTV4200 or pTV400.

To identify the region of DNA required in *cis* for replication, deletions of pTV400 that were unable to replicate in *E. coli* GW125a were transformed into the same strain containing pDER412. A 185-bp DNA fragment common to both pTV4161 and pTV4210 was identified as the region of DNA required in *cis* for plasmid replication (Fig. 1).

The ability of different fragments of pTV100 to displace pDER412 was used to identify the region containing the plasmid incompatibility determinants. From the results in Fig. 1, it is clear that plasmid incompatibility is also located in the 184 bp of DNA common to pTV4161 and pTV4210. However, it is interesting that plasmid pTV4220, in which all three repeated sequences were deleted, retained partial incompatibility with respect to the pTF-FC2 replicon. Since pTV4220 retained that part of the replicon that encodes for the diffusable products required in *trans* for replication, it is most likely the presence or regulation of one of these products that is responsible for the partial incompatibility observed.

Plasmid copy number. Plasmid pTV100 in *E. coli* GW125a was estimated by the hybridization method to have a copy number of 12 to 15 plasmids per chromosome. All plasmids capable of *polA*-independent replication, with the exception of pTV4210, conferred an identical level of ampicillin resistance (800 μg/ml) to *E. coli* GW125a. The presence of pTV4210 resulted in a decrease in resistance to ampicillin (600 μg/ml).

Nucleotide sequence of DNA fragment required in *cis* for replication. The nucleotide sequence of the 329-bp DNA fragment corresponding to the region common to pTV4161 and pTV4200 was determined (Fig. 2). The most notable feature of this region is the presence of three perfectly conserved 22-bp tandemly repeated sequences (nucleotides 9 through 75). There are two sets of complimentary inverted repeat sequences (nucleotides 135 through 152 and 170 through 196) that are able to form potential stem-and-loop structures with ΔGs of −p5.4 and −14.7 kcal/mol, respectively (21). It is not known whether these inverted complimentary repeated sequences have any role in vivo. However, it is interesting that plasmid pTV4210, which appeared to be maintained at a lower copy number than pTV4200, lost one of the second set of complementary inverted repeat sequences.

Comparison of the DNA regions required in *cis* for replication of pTF-FC2 and the IncQ plasmids. The 329-bp DNA sequence of pTF-FC2 and the 370- and 210-bp *Hpa*II fragments of R1162 (12) required in *cis* for replication were

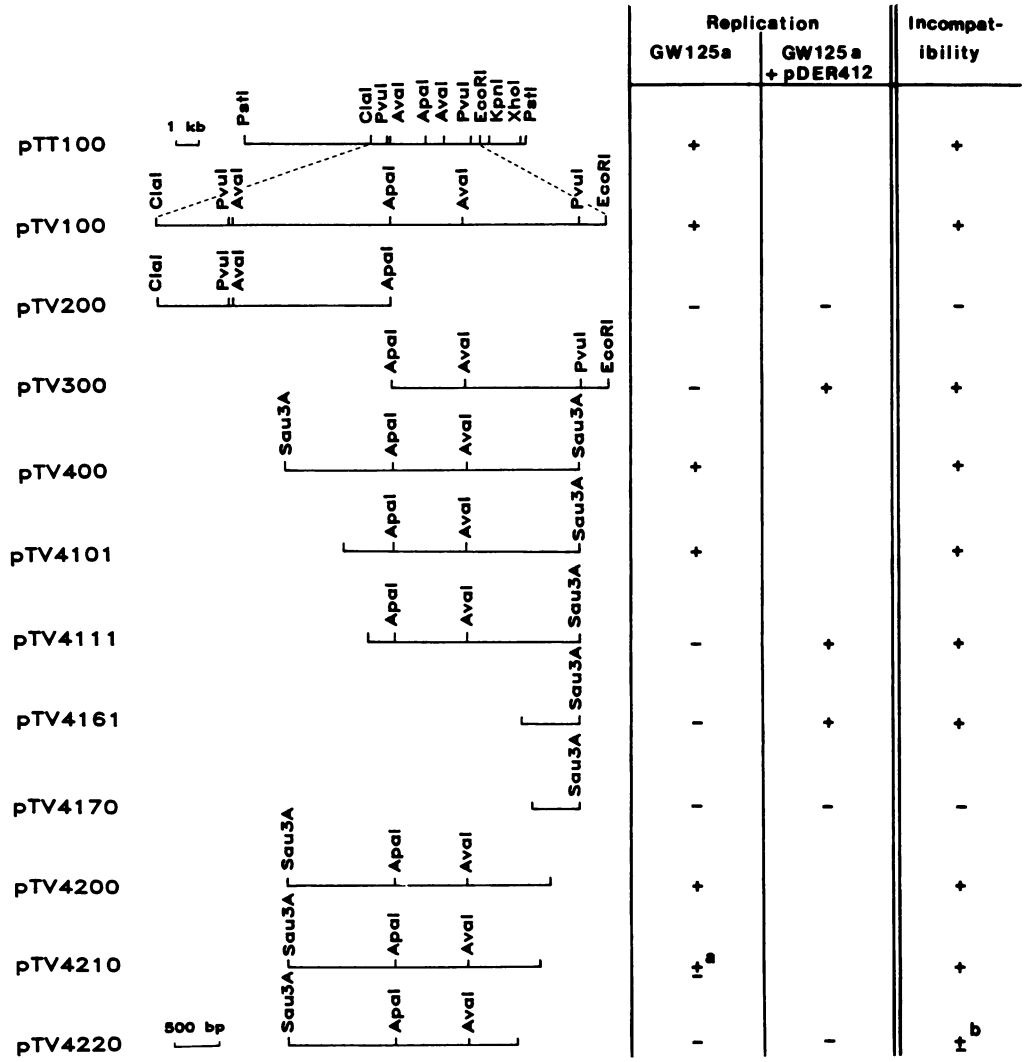

FIG. 1. Restriction endonuclease cleavage map of pTT100 and subclones, showing the identification of the minimal replicon, the region required in *cis* for replication, and the incompatibility determinants. Only pTF-FC2-derived DNA fragments are shown. Superscripts: a, colonies of reduced size; b, partial plasmid incompatibility (37 of 60 colonies were Cm^r).

aligned and compared. Substantial homology was found in the region shown in Fig. 3. The IncQ plasmids had a 60.1% homology (40 of 66 bp) with the pTF-FC2 plasmid in the region of the three repeated sequences. There was 75% homology between the DNA sequences immediately adjacent to the repeated sequences. Beyond this region the two sequences had only limited DNA homology. No further homology between the 329-bp pTF-FC2 DNA sequence and the remainder of the sequence of pTV4161 was found with the R1162 *Hpa*II fragments (results not shown).

Incompatibility between pTF-FC2 and the IncQ plasmid R300B. Since there was a considerable degree of homology between the repeated sequences and adjacent DNA regions of pTF-FC2 and the IncQ plasmid, we examined whether any incompatibility between pTV4161 and pKE462 (Table 1) could be detected. Plasmid pTV4161 carries a 650-bp fragment containing the three repeat sequences and 580 bp downstream. It was tested for its ability to displace pKE462. All 60 colonies tested were Tc^r, indicating that these two plasmids were compatible.

DISCUSSION

There are several similarities between the 12.4-kb cryptic *T. ferrooxidans* plasmid pTF-FC2 and the 8.9-kb IncQ plasmids R1162, RSF1010, and R300B. Both types of plasmid have a broad host range and are *polA* independent, and they have comparable copy numbers in *E. coli*. There are, however, some substantial differences. In RSF1010 the minimum replicon is spread over approximately 5.5 kb of DNA and is interspersed with regions that are not required for replication (1). The pTF-FC2 replicon is more compact and is confined to a 2.1-kb DNA fragment. Whether the broad-host-range properties of the parent plasmid are contained on this DNA fragment is under investigation.

A 184-bp region of plasmid DNA that is required in *cis* when the parent plasmid pDER412 is present in *trans* has been identified and sequenced. Nucleotide sequence comparisons between this fragment and the origin of replication of the IncQ plasmids R1162 and RSF1010 indicated that

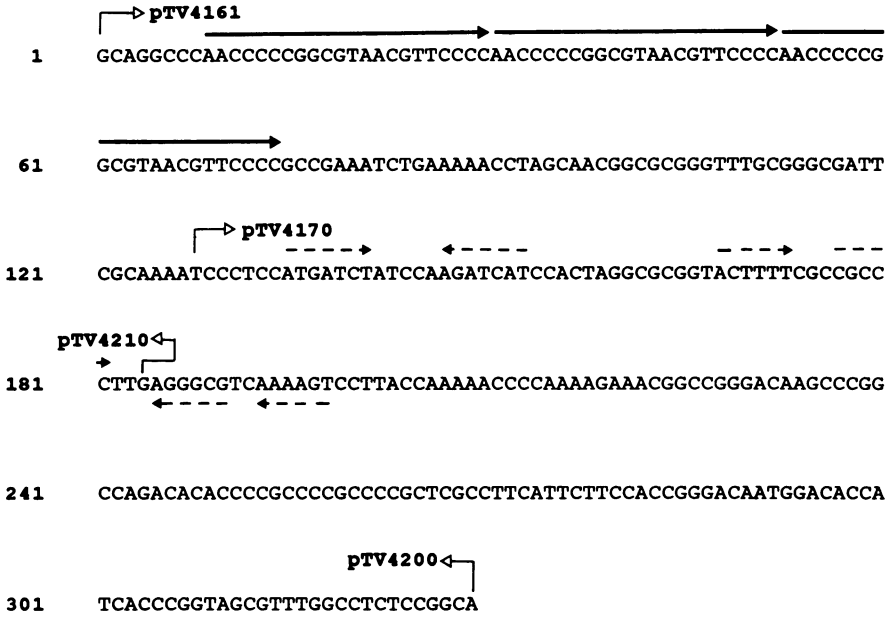

FIG. 2. Nucleotide sequence of the fragment of pTF-FC2 encompassing the region of DNA required in *cis* for replication. Solid arrows indicate the 22-bp repeated sequences, and broken arrows indicate complementary inverted repeated sequences.

pTF-FC2 has three consecutive 22-bp tandem perfectly conserved directly repeated sequences, whereas the IncQ plasmids have three and one-half perfectly conserved 20-bp repeated sequences (13, 15). If the two sets of repeated sequences including the two spacer nucleotides of the IncQ plasmids are aligned, there is 60% homology between them. From this comparison, it may be argued that the unit length of the repeated sequences of the IncQ plasmids is in fact 22 bp and that the first 2 bp are imperfectly conserved.

In R1162 two distinct domains, contained on adjacent 370- and 210-bp *Hpa*II fragments, are required in *cis* for plasmid replication (7). These domains are able to direct plasmid replication even when the distance between them is increased. The 184-bp sequence of pTF-FC2 has extensive homology with a sequence contained entirely within the 370-bp *Hpa*II fragment. Plasmid pTF-FC2 therefore does not appear to have a requirement for a domain equivalent to that on the 210-bp *Hpa*II fragment of the IncQ plasmids.

The 75% DNA homology between the 115 bp of DNA immediately adjacent to the repeated sequences of pTF-FC2 and the *oriV* of the IncQ plasmids is striking. The absence of homology outside this region may indicate structural conservation between *oriV* regions of the small broad-host-range plasmids.

The repeated sequences of the IncQ plasmids have been shown to be responsible for plasmid incompatibility and copy number control (10, 13). The 184-bp DNA sequence from pTF-FC2 containing the three repeats has also been shown to be involved in plasmid incompatibility. Although the repeated sequences of the two plasmids are 60% homol-

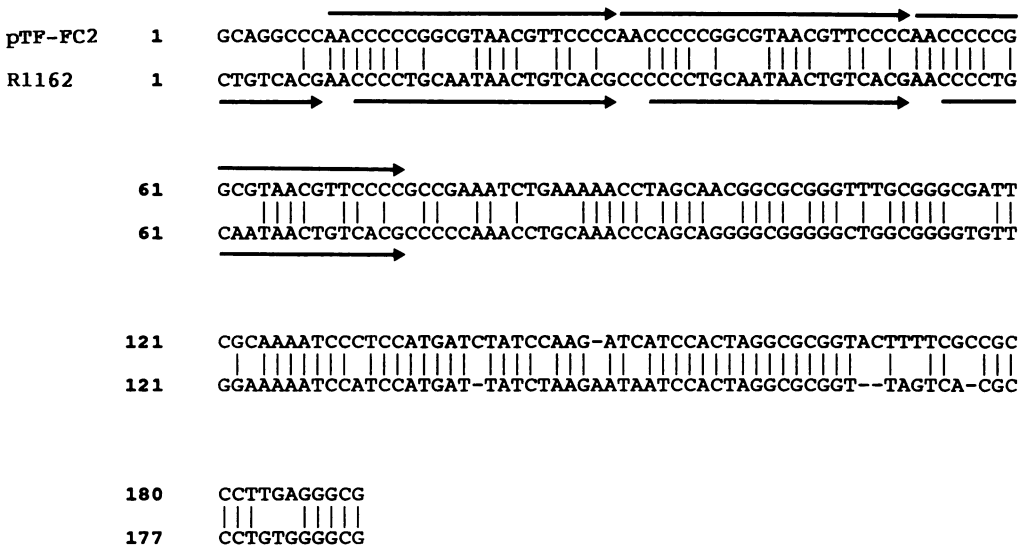

FIG. 3. Comparison of nucleotide sequence homology between the repeated sequences and adjacent regions of pTF-FC2 and R1162.

ogous, no incompatibility between the two sets of repeated sequences was detected.

The similarities between the nucleotide sequences of the pTF-FC2 origin of replication and those of the IncQ plasmids may reflect the features of a class of broad-host-range replicons to which both these plasmids belong. Whether the similarities extend to the diffusible products that are required in *trans* remains to be determined. Nucleotide sequencing of the rest of the basic replicon and *trans* complementation studies between pTF-FC2 and the IncQ plasmids are currently in progress.

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