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Cloning of a *Thiobacillus ferrooxidans* Plasmid in *Escherichia coli*

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Three separate plasmids of 6, 7, 16, and >23 kilobases were purified from a single clone of *Thiobacillus ferrooxidans* ATCC 33020 grown in the presence of uranium. The 6.7-kilobase plasmid (pTf1) was cloned separately into the *Hin*dIII or *Bam*HI site of *Escherichia coli* plasmid pBR322. Restriction maps of the recombinant plasmids, termed pTf100 and pTf110, respectively, were constructed, creating potential cloning vehicles for exchanging genetic information between *E. coli* and *T. ferrooxidans*. Evidence from restriction enzyme analysis and Southern blot DNA-DNA hybridization indicates that the three native plasmids share little sequence homology.

Thiobacillus ferrooxidans and some related thiobacilli are acidophilic, chemolithotrophic bacteria that derive energy and reducing power from the oxidation of reduced or partially reduced sulfur compounds and, in some cases, iron compounds. The iron-oxidizing thiobacilli can grow in the presence of high concentrations of many metallic ions (1), but the genetic and biochemical bases of these metal resistances have not been determined. Since resistance to several metals, such as cadmium, mercury, and arsenic, is plasmid encoded in other species of bacteria (3, 12, 15, 16), it has been speculated that metal resistance might be similarly encoded on plasmids in thiobacilli (2, 8, 16). The majority of *T. ferrooxidans* strains that have been examined contain plasmids (10, 11). In one strain of *T. ferrooxidans* the disappearance of a ca. 18-kilobase (kb) plasmid was positively correlated with the disappearance of uranium resistance (P. A. W. Martin, P. R. Dugan, and O. H. Tuovinen, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, I34, p. 92). Such circumstantial evidence is not sufficient to establish a causal relationship. We cloned a 6.7-kb plasmid from a strain of *T. ferrooxidans* (ATCC 33020; originally isolated from a uranium mine [17]) into *Escherichia coli* plasmid pBR322 to obtain large quantities of an otherwise difficult-to-isolate plasmid for subsequent structural and genetic analyses and to construct a suitable cloning vehicle that will replicate and be expressed in both *E. coli* and *T. ferrooxidans*.

T. ferrooxidans ATCC 33020 was obtained from the American Type Culture Collection. A clone was isolated on 9K-$FeSO_4$ agarose plates essentially by the method of Manning (9) and was cultured in 9K medium (18) with 0.16% (wt/vol) uranyl nitrate at 30°C. Plasmids were isolated from a cleared lysate of cells essentially as described previously by Martin et al. (11). Plasmids were purified by buoyant density centrifugation in CsCl-ethidium bromide gradients (4) and visualized by electrophoresis in a 0.8% agarose gel (Fig. 1).

There were three visible covalently closed circular species of plasmids (Fig. 1) which we termed pTf1, pTf2, and pTf3. *Hin*dIII restriction of pTf1 and pTf2 generated single linear forms which were 6.7 and 16 kb, respectively. Comparison with its covalently closed circular form (Fig. 1, lane 2) indicated that pTf3 is also cleaved with *Hin*dIII, resulting in a linear fragment of greater than 23 kb.

The three plasmids can be distinguished by their different restriction patterns. For example, pTf1 was not cleaved by *Sal*I, *Pvu*II, or *Bam*HI, pTf2 was cleaved once by *Pvu*II and twice by *Bam*HI (the second *Bam*HI fragment is ca. 1.5 kb and was not visible on this gel), and pTf3 was cleaved by *Sal*I.

This comparative restriction analysis demonstrates for the first time that a purified strain of *T. ferrooxidans* contains multiple plasmids. Although previous workers (10, 11) have observed multiple plasmid bands in agarose gels, it is possible that the plasmids arose from a mixed population of strains. Alternatively, the observed plasmids could have been open and closed forms of multimers of the same plasmid.

Plasmid DNA from *T. ferrooxidans* was cleaved with *Hin*dIII, and the fragments were separated by agarose gel electrophoresis. The band corresponding to *Hin*dIII-cleaved pTf1 was excised (13), and the DNA was recovered from the gel and ligated into the *Hin*dIII site of plasmid pBR322. After transformation into *E. coli* C600, clones harboring recombinant plasmids were screened by testing for insertional inactivation of the tetracycline resistance gene (5). Several such clones were identified, and plasmids were isolated from them by a rapid procedure (7). A clone containing the expected 6.7 kb of pTf1 and 4.36 kb of pBR322 was identified, and large quantities of this plasmid, termed pTf100, were purified by density centrifugation in CsCl-ethidium bromide gradients (4, 6). Purified plasmid DNA was analyzed, using a number of restriction enzymes. The pTf1 component lacked the following restriction enzyme sites: *Bam*HI, *Eco*R1, *Eco*RV, *Sal*I, *Pst*I, *Bcl*I, *Kpn*I, *Xba*I, *Mlu*I, *Sac*I, and *Pvu*II but contained sites for *Bgl*I, *Bgl*II, *Cla*1, and *Sma*I (and *Ava*I, which recognizes a subset of *Sma*I sites), as well as the *Hin*dIII site used for cloning. Double digests or partial digests of pTf100 were used to map the restriction sites found in the pTf1 component (Fig. 2A).

The same disposition of restriction enzymes sites was found in the native pTf1 plasmid (data not shown). Hence, these sites have not been modified in *T. ferrooxidans*, for example, by methylation or glycosylation.

pTf100 was nick translated and used to probe Southern blots of native plasmids isolated from *T. ferrooxidans* ATCC 33020 (14, 19) (Fig. 3). As expected, pTf100 hybridized extensively to pTf1, establishing the source of the cloned DNA. A limited amount of hybridization to the closed circular form of pTf2 was observed. The nature and extent of this hybridization remain to be determined by more detailed studies. It should be noted that Fig. 3B represents an

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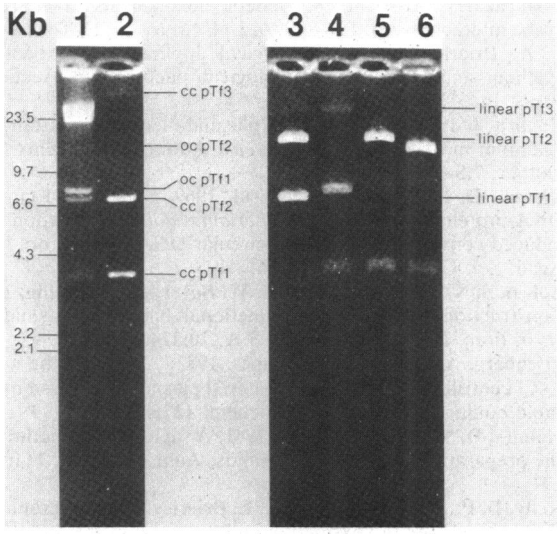

FIG. 1. Agarose gel electrophoresis of plasmids isolated from *T. ferrooxidans* ATCC 33020. Plasmids were visualized either directly from a cleared cell lysate (11) (lane 1) or after additional purification by CsCl-ethidium bromide centrifugation (lanes 2 through 6). Purified plasmids were cleaved with *Hin*dIII (lane 3), *Sal*I (lane 4), *Pvu*II (lane 5), or *Bam*HI (lane 6). Fragment sizes in kbp from a λ phage digested with *Hin*dIII are shown at the left. oc, Open circular plasmids; cc, closed circular plasmids. Restriction enzymes were obtained from either New England Biolabs or Bethesda Research Laboratories and used according to the instructions of the manufacturer. DNA was resolved on 0.8% agarose gels (SeaKem HGT) in 40 mM Tris–5 mM sodium acetate–1 mM EDTA (pH 7.9) at 2 V/cm for 16 h. DNA was visualized by staining with 0.1 μg of ethidium bromide per ml and photographed with a model 800 Polaroid Land camera, using type 47 Polaroid film and a no. 25A red filter.

overexposure of the autoradiograph of the Southern blot, so that hybridization to the open circular form of pTf1 is visible on the Southern blot even though the open circular form is not visible on the ethidium bromide-stained agarose gel. Conversely, the extent of hybridization to the closed circular form of pTf2 is about the same as to the open circular form of pTf1, even though the mass of pTf2 present compared to that of the open circular form of pTf1 is much more as judged by the intensity of ethidium bromide staining. Thus, the degree of homology of pTf100 with pTf2 is far less than with pTf1, even though the intensities of the bands on the autoradiogram are about the same.

No hybridization of pTf100 to the closed circular form of pTf3 could be detected. This does not preclude the possibility of some sequence homology between these plasmids. Some homology may exist but cannot be detected by the present experiment due to the limited quantity of pTf3 on the gel. Further analysis may have to await the isolation and cloning of pTf3.

Since cloning at the *Hin*dIII site of pTf1 could inactivate the origin of replication or some important genetic marker, pTf1 was also cleaved with *Bgl*II and cloned into the *Bam*HI site of pBR322. This can be accomplished because the core of the restriction site for *Bgl*II hybridizes to the core of the *Bam*HI site. A restriction map of the resulting plasmid (pTf110) is shown in Fig. 2B. pTf100 and pTf110 provide suitable vehicles for generating large quantities of pTf1 for genetic and biochemical studies. Furthermore, both recombinant plasmids carry the β-lactamase gene of pBR322, which confers resistance to ampicillin. Ampicillin is effective at pH 4.3 and hence might be a useful selective marker in transformation studies of thiobacilli grown in thiosulfate at pH 4.3. It is hoped that one of the configurations of pTf1 in either pTf100 or pTf110 will retain a functional origin for

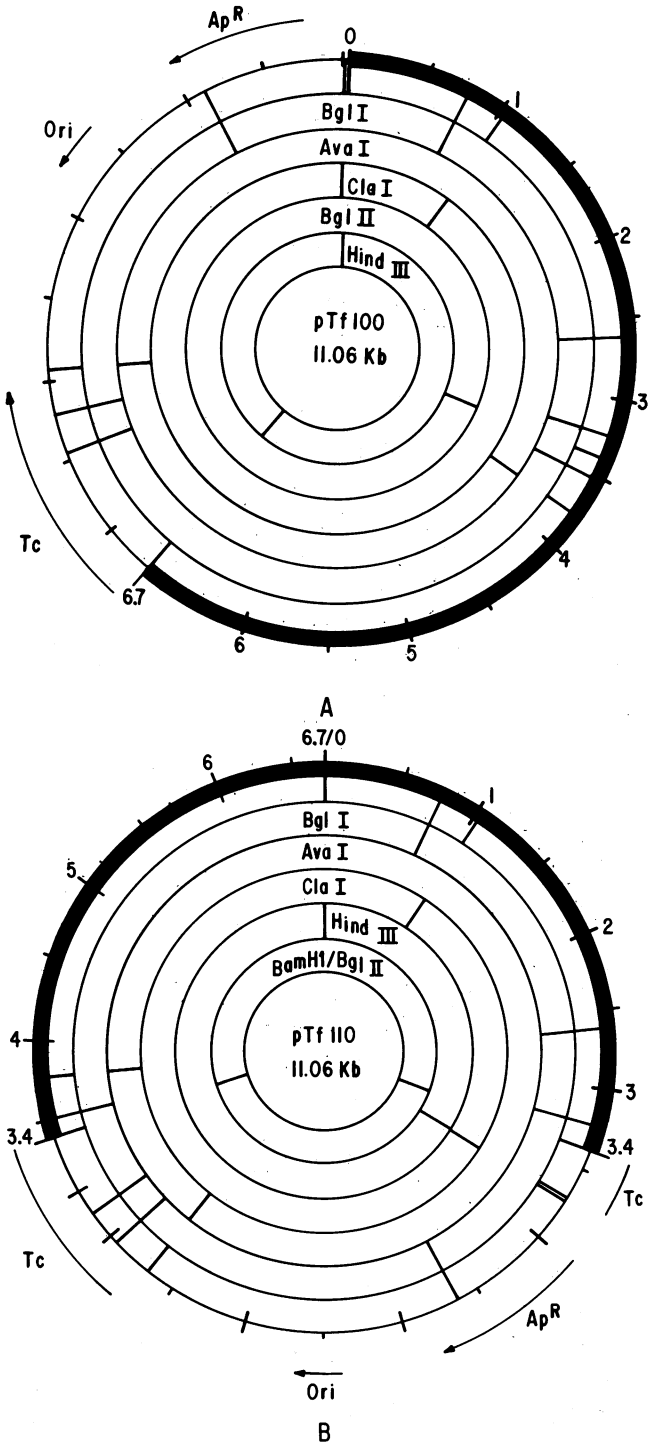

FIG. 2. Restriction enzyme maps of (A) pTf100 and (B) pTf110. Thick line, pTf1 component; thin line, pBR322 component. Coordinates are in kb. Tc, Region encoding tetracycline resistance, not functionally expressed in pTf100 or pTf110; Ap^R, β-lactamase gene encoding ampicillin resistance; Ori, origin of replication region in pBR322.

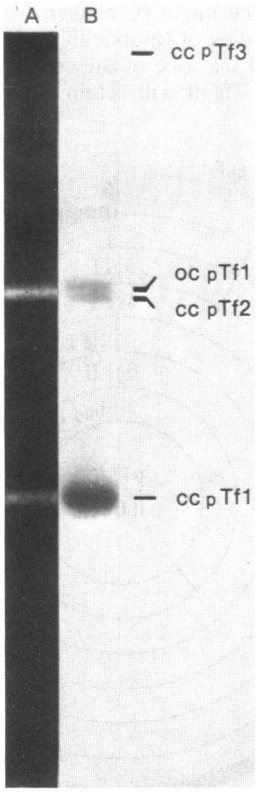

FIG. 3. Southern blot DNA-DNA hybridization analysis of purified native plasmids of *T. ferrooxidans* hybridized with ^{32}P-labeled, nick-translated pTf100 DNA. (A) Ethidium bromide-stained agarose gel; (B) autoradiograph. oc, Open circular plasmids; cc, closed circular plasmids.

replication in *T. ferrooxidans* and hence will be of value to shuttle genetic information between *E. coli* and *T. ferrooxidans*. This remains to be determined.

Our laboratory has adopted a nomenclature for *T. ferrooxidans* plasmids in which the initials pTf are followed by a plasmid number. Recombinant plasmids constructed from native plasmids receive the same initials and first digits of the native plasmids from which they were derived. For example, pTf1 is a native *T. ferrooxidans* plasmid from which the two recombinant plasmids pTf100 and pTf110 were constructed. We think this system is much more informative than using the initials of investigators or institutions, since it identifies both the species of origin and the relatedness of native plasmids and their recombinant derivatives.

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