Physical Mapping and Characterization of a Catabolic Plasmid from the Deep-Subsurface Bacterium *Sphingomonas* sp. Strain F199

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A supercoiled 180-kb plasmid, pNL1, has been isolated from the deep-subsurface, chemoheterotrophic *Sphingomonas* sp. strain F199, and a physical map was generated. Analysis of a pNL1-derived cosmid library indicated that catechol 2,3-dioxygenase activity was linked to two distinct regions of the plasmid. Thus, the genes for aromatic catabolism in this *Sphingomonas* strain are, at least in part, plasmid encoded.

Surface soil and aquatic bacteria capable of using aromatic hydrocarbons as carbon and energy sources are relatively common and make in situ bioremediation of petroleum hydrocarbon-contaminated soils and shallow sediments using indigenous microorganisms feasible. Although many of these catabolic pathways have been described for bacteria residing in soil and near-surface environments (23), relatively little is known about the physiology of bacteria in the deep subsurface.

A subsurface organism, strain F199, isolated from uncontaminated sediments 407 m below the soil surface at the Department of Energy Savannah River Site has the ability to use toluene, all xylene isomers, *p*-cresol, naphthalene, salicylate, and benzoate as sole carbon and energy sources (7). This isolate has been classified in the genus *Sphingomonas* on the basis of 16S rRNA gene sequence and fatty acid analysis (8). Although the members of the genus *Sphingomonas* are known to have extensive abilities to degrade xenobiotic compounds (15, 19, 25), little is known about the genes involved or their organization.

Strain F199 was previously shown to harbor two large plasmids of over 100 kb (7). Because catabolic genes are often found on plasmids in aerobic, gram-negative bacteria (22, 26, 27), the plasmids in F199 were of particular interest.

**Megaplasmid isolation.** A number of approaches (1, 9, 11, 24) were used in attempts to purify F199 plasmid DNA, with minimal success, possibly because of copurification of polysaccharides produced by this microorganism. Better yields of plasmid were obtained by the method of Casse et al. (3); for this, F199 cells were grown in King’s medium B (13) at 30°C in shaking cultures. To remove small fragments of DNA, the precipitated DNA resulting from alkaline lysis purification was dissolved in 400 μl of 10 mM Tris-HCl–1 mM EDTA (pH 7.5) (TE) and further purified by centrifugation (2 h at 100,000 × g) through a sucrose gradient consisting of 2.5-ml layers of 40, 30, 20, and 10% (wt/vol) sucrose in TE. Fractions containing pure plasmid were pooled and concentrated by use of Centri-100 (Amicon Inc., Beverly, Mass.) spin filters or by ethanol precipitation. These procedures resulted in an essentially pure preparation of a single large plasmid termed pNL1.

Previous data (7) suggested that F199 contained two megaplasmids. While working out plasmid isolation procedures, it became apparent that the two bands seen earlier were different forms of pNL1. Nonetheless, recent results (data not shown) indicate that F199 indeed has a second, even larger plasmid, but this replicon could not be isolated by the methods described above.

**Megaplasmid characterization.** In pulsed-field gel electrophoresis (PFGE), pNL1 DNA, as isolated, migrated near the linear 582-kb marker (Fig. 1). Digestion of the purified pNL1 with SpeI yielded a single restriction fragment of 180 kb (Fig. 1), consistent with the plasmid becoming linearized. In addition, purified pNL1 DNA nicked by exposure to UV light was unable to enter the gel (data not shown). Since in PFGE the supercoiled forms of large plasmids migrate more slowly than the linear forms (14, 16) and large relaxed circles do not enter the gel (14), these data suggested that pNL1 is a supercoiled molecule in F199.

**Physical map of pNL1.** To provide a framework for genetic characterization, a physical map of pNL1 was constructed. The plasmid was digested with several restriction enzymes. The plasmid was cut twice by *Ase*I. The relative positions of these sites (Fig. 2) were determined by double digests.

**NotI** digestion of pNL1 resulted in 14 bands ranging in size from 600 bp to 45 kb; *SspI* digestion resulted in 12 fragments

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FIG. 1. PFGE of pNL1 plasmid preparations. The DNA was electrophoresed through a 1% agarose gel in 0.5× Tris-borate-EDTA at 200 V with pulse durations that increased from 15 to 30 s over 20 h. Lane 1, lambda ladder size standards; lane 2, pNL1 as purified from F199; lane 3, pNL1 cut with SpeI. The positions of the various DNA forms are indicated on the right.
between 1.8 and 51 kb in size. Other restriction enzymes with recognition sequences of 6 or more bp (BamHI, DraI, EcoRI, HindIII, PvuII, SgfI, SmaI, and SrfI) generated too many bands to be useful for initial mapping.

The NotI and SspI fragments were ordered by (i) double digests of either NotI or SspI with the rare cutters SpeI, XbaI, and AseI; (ii) digestion of purified NotI fragments with SspI; (iii) digestion of purified SspI fragments with NotI; (iv) hybridization of SspI digests with purified NotI fragments; (v) hybridization of NotI digests with purified SspI fragments; (vi) partial NotI or SspI digests followed by hybridization with specific fragments; and (vii) PCR of intact pNL1 using primers derived from the sequenced ends of NotI fragments (Fig. 2).

**Analysis of pNL1 sequences for catechol 2,3-dioxygenase (C23O) activity.** To provide a tool for determining what genes reside on pNL1, a cosmid library of the megaplasmid was constructed. Fragments from a partial Sau3AI digest were cloned into the vector sCos1 (6), with an average insert size of 40 kb. The bacterial host for the library was *Escherichia coli* DH5α. Approximately 300 cosmid clones were isolated. Digestion of these clones with NotI or SspI demonstrated relatively uniform coverage of pNL1 by the cosmids.

Cells containing each cosmid were spotted onto Luria-Bertani agar, grown overnight, and sprayed with 1% catechol solution in methanol. The plates were allowed to incubate for 1 h to screen for C23O activity. The appearance of a bright yellow color was indicative of the meta cleavage product of catechol. Forty cosmid clones tested positive without any prior exposure of the colonies to aromatic compounds. C23O activity was confirmed by analysis of cell extracts (data not shown). Because this activity is often found in the degradation of a number of aromatic compounds, the hypothesis that catabolic genes would be encoded on the F199 plasmid was confirmed. Furthermore, the common occurrence of this gene as part of a larger operon encoding other genes involved in biodegradation suggests that additional genes will be found nearby.

The region of pNL1 encoding C23O activity was localized by restriction mapping of 26 cosmid clones capable of expressing this activity and 85 lacking this activity, followed by alignment of these cosmids with the pNL1 map. Diagrams of representative clones (Fig. 3) illustrate that two separate regions on pNL1 encode C23O activity. For example, cosmids 18 and 6 do not overlap, but both tested positive for C23O activity. The unique sequences in cosmid 18 that are not present in clone 230, which tested negative, define the boundaries of one region. Similarly, the area from the left end of cosmid 6 to the left end of cosmid 75 defines another region with C23O activity. Although only representative cosmids are shown, the two regions defined are consistent with all analyzed cosmids.

**Discussion.** To our knowledge, this is the first linkage of a catabolic function to a plasmid in the genus *Sphingomonas*, although biodegradative functions of *Sphingomonas* isolates have been described previously (10, 17, 20, 21). Previous studies have shown that certain degradative gene clusters are located on plasmids and transposable elements in some bacteria (4, 18, 27, 28). Analysis of the 180-kb plasmid pNL1 identified C23O genes in two regions of the plasmid, but the regulation and substrate specificity of these two C23O genes on pNL1 are currently unknown. Multiple copies of homologous or nonhomologous C23O structural genes have previously been identified on TOL plasmids in *Pseudomonas* strains isolated from soil (5). Also, two nonhomologous C23O genes were present on plasmid pWW15 in *Pseudomonas putida* MT15 (12); the substrate specificities of the two C23O genes were distinct, as was their regulation.
C23O and related meta ring cleavage enzymes function in the catabolic pathways for the biodegradation of many aromatic compounds, including naphthalene, toluene, xylene, and biphenyl. All of these aromatic compounds are degraded by F199; hence, the pathways for catabolism of one or more of them may be encoded on pNL1. Further analysis of pNL1 will likely reveal other enzymes involved in aromatic compound degradation, since catabolic plasmids such as pWW0 usually encode entire operons and their regulatory elements (2). It is interesting, however, that neither pNL1 nor F199 genomic DNA shared significant homology with pWW0 or NAH7 catabolic plasmids (7), which code for the catabolism of toluene and naphthalene, respectively. Additional genetic and biochemical analysis of F199 and the pNL1 plasmid will provide important insights into the nature of aromatic compound degradation genes from the isolated deep subsurface environment.

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REFERENCES