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Gene transfer from a bacterium injected into an aquifer to an indigenous bacterium

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Abstract

Two novel 3-chlorobenzoate-degrading bacteria were previously isolated from an aquifer in which no such bacteria could be enriched prior to the introduction of the 3-chlorobenzoate-degrading strain, *Pseudomonas* sp. B13. To understand the origin of 3-chlorobenzoate-degrading genes in the two novel isolates, the 16S ribosomal RNA, *clc*D (dienelactone hydrolase) and *clc*A (chlorocatechol oxygenase) genes from these bacteria were amplified and sequenced. The partial 16S rRNA gene sequences and REP-PCR patterns showed that these two novel isolates were identical but differed from strain B13. Phylogenetic analyses revealed that the novel isolates were closely related to *Alcaligenes eutrophus* in the beta subclass of the *Proteobacteria*, whereas strain B13 was related to *Pseudomonas aeruginosa* and *P. mendocina* in the gamma subclass of the *Proteobacteria*. In contrast, the *clc*D and *clc*A gene sequences were identical on strain B13 and these two isolates, indicating that the 3-chlorobenzoate-degrading genes were transferred from strain B13 to these isolates. What cannot be established is when this transfer occurred.

Keywords: gene transfer, molecular evolution, 3-chlorobenzoate degradation, phylogeny

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Introduction

In a previous study, two novel 3-chlorobenzoate-degrading bacteria were isolated from a sand and gravel aquifer on Cape Cod, Mass into which the 3-chlorobenzoate-degrading bacterium, *Pseudomonas* sp. strain B13, was injected 14.5 months earlier (Thiem *et al.* 1994). Using the catabolic gene, *clc*D, which codes for dienelactone hydrolase, as a probe, identical restriction patterns were observed between these two isolates and strain B13. However, when the 23S gene was used as a probe, the restriction patterns of these two isolates were identical but different from that of *Pseudomonas* sp. strain B13. Furthermore, these two isolates showed no hybridization to the random-cloned, strain B13-specific fragment (Thiem *et al.* 1994). These results indicated that these two isolates were different from the injected bacterium, *Pseudomonas* sp. strain B13, but that they may have similar dienelactone hydrolase genes.

There are two possible explanations for the origin of 3-chlorobenzoate activity in the new isolates: one is that the 3-chlorobenzoate catabolic pathway genes have been transferred from B13 to indigenous bacteria, and the other is that indigenous 3-chlorobenzoate degrader(s) carry a highly homologous *clc*D gene on the same restriction fragment. Although the failure of enriching 3-chlorobenzoate-degrading micro-organisms from the aquifer before injection (Krumme *et al.* 1994; Thiem *et al.* 1994) suggested that the first explanation may be more likely, direct evidence was lacking. We tested these two hypotheses by comparing the nucleotide substitution rates of the 3-chlorobenzoate catabolic genes from B13 and the two isolates with a reference gene essential to organisms; we used the 16S rRNA gene. If the 3-chlorobenzoate catabolic genes were transferred from B13, the number of nucleotide substitutions in the 3-chlorobenzoate catabolic genes between B13 and these two isolates will be significantly less than the expected nucleotide substitutions of the 16S rRNA genes (assuming that the organisms would have at least some 16S rRNA sequence differences).

In order to resolve this issue, partial nucleotide sequences of *clc*D, *clc*A and 16S rRNA genes from strain B13 and these two isolates were determined and compared with gene sequences in databases. Since the *clc*D and *clc*A gene sequences among strain B13 and the two environ-

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mental isolates were identical and the 16S rRNA genes were not, gene transfer must have occurred.

Materials and methods

Bacterial strain and DNA isolation

Pseudomonas sp. strain B13 was isolated from sewage in Germany (Dorn *et al.* 1974) and the two novel 3-chlorobenzoate-degrading isolates were isolated by Thiem *et al.* (1994). B13 had been injected to a density of 10^5 organisms/g dry weight of sediments in an injected zone of 0.9 m in diameter (Krumme *et al.* 1994). The novel isolates were isolated after enrichment for 4 weeks on 3-chlorobenzoate liquid medium under continuous shaking at 200 r.p.m. The medium was inoculated with 10 g aquifer core solids drilled from the injected zone 8 m below the surface and 14.5 months after injection of B13 (Thiem *et al.* 1994). For convenience, these two novel isolates were designated as 3CB-1 and 3CB-2, respectively. *Pseudomonas* sp. strain B13, 3CB-1 and 3CB-2 were grown at 30 °C on M9 medium supplemented with trace minerals and 5-mM 3-chlorobenzoate. Isolation of total DNA from each bacterial strain was carried out by an SDS-based lysis method (Zhou *et al.* 1995).

Colony REP-PCR

The colony REP-PCR was performed as described by de Bruijn (1992). Each isolate was grown on R2A medium plate for 24–48 h, and then a small amount of cells was resuspended in a 25 μL of PCR mixture (de Bruijn 1992). After PCR amplification, one-third of the amplified products were separated on 1.5% agarose gel.

PCR amplification of clc *and 16S rRNA genes*

The *clc* gene-specific oligonucleotide primers were designed using the gene sequence from *Pseudomonas* sp. strain B13 (Frantz *et al.* 1987) and the OLIGO program (Rychlick & Rhoads 1989). The 5′ and 3′ primers for the *clc*D gene were 5′-GACGGGCATACATTCGGCGC-3′ and 5′-GGCACTCGCCACATAGCCCG-3′, which complement the regions of 34-53 and 629-610 of the *clc*D gene, respectively. The 5′ and 3′ primers for *clc*A gene, which encodes catechol oxygenase II, were 5′-TTGCTGGACAAGCGCGTCAC-3′ and 5′-CGGCCCCTCGTAGGGGATCT-3′, which complement the regions of 58-77 and 513-494 of the *clc*A gene. The oligonucleotides used for amplifying 16S rRNA genes were described previously (Zhou *et al.* 1995). All of the oligonucleotides were synthesized by the Macromolecular Structure, Sequencing and Synthesis Facility at Michigan State University.

All stocks for PCR amplification were made and procedures were performed with the precautions suggested by Kwok & Higuchi (1989). Nearly full length 16S rRNA genes were amplified as described previously (Zhou *et al.* 1995). For *clc*D gene amplification, the template DNA was added to a standard polymerase chain reaction mix containing 1 × *Taq* polymerase buffer, 2.5 units *Taq* polymerase (Promega, Madison, WI), 20 nmol dNTPs and 100 pmoles of each primer in a 100-μL reaction. Each reaction mixture was heated to 92 °C for 30 s, which was followed 30 cycles of 94 °C for 30 s, 60 °C for 1 min and 72 °C for 2 min. Samples were then subjected to a 7 min incubation at 72 °C before being stored at 4 °C. The amplification conditions for *clc*A gene were the same as those for *clc*D gene except the annealing temperature was 62 °C. Temperature cycling was performed in a programmable temperature cycler (GeneAmp PCR System 9600, Perkin Elmer Corp., Norwalk, CT).

DNA sequencing and sequence analysis

The DNA sequences of the 16S rRNA gene and *clc* genes from each bacterial isolate were determined directly from both directions using the PCR-amplified DNA as the sequencing template. The amplified PCR products were purified by using Wizard™ PCR Preps DNA purification system (Promega, Madison, WI) according to the manufacturer's instructions. DNA sequences were determined with automated fluorescent *Taq* cycle sequencing using the ABI Catalyst 800 and ABI 373 A Sequencer (Applied Biosystems, Forester City, CA). About 100 ng of the purified DNA was used for one automated fluorescent sequencing reaction. The sequencing primers for 16S rRNA gene used in this study spanned *E. coli* 16S rRNA gene positions as follows: 519-533 (F, forward), 787-802(F), 1115-1100 (R, reverse) and 802-787 (R). The same primers used for PCR amplification were used for sequencing *clc*D and *clc*A genes from each isolate.

The phylogenetic analysis was carried out as described previously (Zhou *et al.* 1995) except that the maximum likelihood tree was constructed using fastDNAml from RDP (Larsen *et al.* 1993) instead of DNAML from PHYLIP (Felsenstein 1989). The number of nucleotide substitutions was calculated with Nei & Gojobori's (1986) unweighted pathway method using the computer program kindly provided by Dr A. Hughes (Pennsylvania State University at University Park).

Plasmid detection and Southern blotting

Individual isolates were cultured in 3-chlorobenzoate broth medium, harvested and lysed as described by Kado & Liu (1981) with some modifications (Ka *et al.* 1994). Two bacterial strains which contain plasmids, *Azoarcus tolulyticus* Td-3 (Fries *et al.* 1994), and strain 2811p (Ka & Tiedje

1994), were used as positive controls. The DNA samples (120–140 μL) obtained from each cell lysate was subjected to electrophoresis in an 0.8% agarose gel to separate circular plasmid DNA from linear fragments of chromosomal DNA. Under the lysis conditions used, the chromosomal DNA is sheared down and migrates faster than circular plasmid DNA. Following gel electrophoresis, the DNA was transferred to GeneScreen Plus nylon membrane (Dupont, Boston, MA) by using 0.4-M NaOH and 0.6-M NaCl solution and fixed onto membranes by drying at room temperature overnight. The membrane was prehybridized with 8 mL of prehybridization solution containing 1 x HSB (For 5 x HSB: 3% PIPES, 3-M NaCl, 20-mM EDTA, pH 6.8), 10% Denhardt III (For 100% Denhardt III: 2% BSA, 10% SDS, 2% Ficoll 400, 2% PVP360 and 5% $Na_4P_2O_7$) and 1 mg denatured herring sperm DNA per millilitre in a plastic box, which was then placed in a shaker (Lab-Line Orbit Environ-Shaker, Lab-Line Instruments, Inc., Melrose Park, IL) at 100 r.p.m. at 65 °C for 12–16 h. The prehybridization solution was removed, and the membranes were hybridized with 5 mL of fresh hybridization solution which consisted of 1 x HSB, 10% Denhardt III, 1 mg of denatured herring sperm DNA per millilitre and the radiolabelled gene probe. The gel purified 615-bp gene fragment amplified from the *clc*D gene was used as probe. A 25–50-ng probe DNA was labelled with [-^{32}P]CTP using T7 QuickPrimer™ kit (Pharmacia, Piscataway, NJ) according to the manufacturer's instructions. After hybridization, the blots were washed at 65 °C for 30 min, once with 2 x SSC plus 1% SDS and once with 0.1 x SSC plus 1% SDS. Blots were exposed for 7 days at –70 °C with intensifying screens.

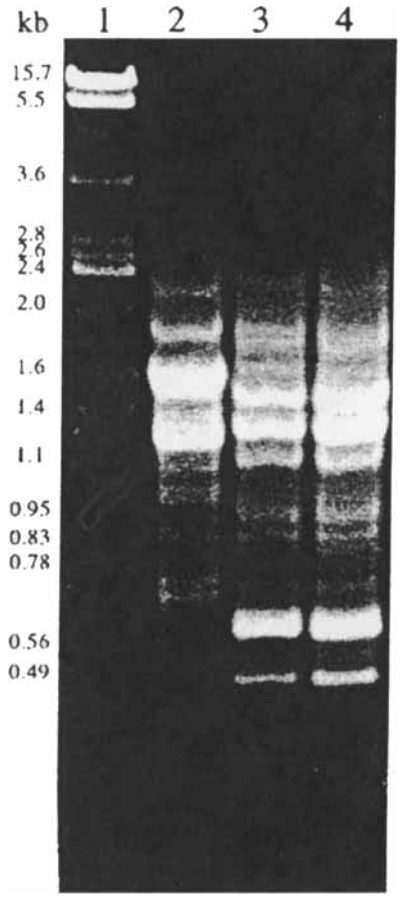

Fig. 1 REP-PCR patterns of isolates. Lane 1, *Bam*HI-, *Eco*RI- and *Hin*dIII-digested lambda DNA standard; Lane 2, *Pseudomonas* sp. strain B13; Lane 3, 3CB-1; Lane 4, 3CB–2.

Nucleotide sequence accession numbers

The accession numbers for the nucleotide sequences of 16S rRNA genes in GenBank are: *Pseudomonas* sp. B13, L40412; 3CB-1, L40413.

Results

To determine the evolutionary relationships among the novel aquifer isolates, strains 3CB-1 and 3CB-2, and the injected strain B13, both REP-PCR patterns and the partial 16S rRNA gene sequences of these organisms were analysed. The REP-PCR patterns were identical among 3CB-1 and 3CB-2 but different from that in strain B13 (Fig. 1). While 3CB-1 and 3CB-2 showed 100% similarity of the partial 16S RNA gene sequences, only 81% similarity was observed between these two isolates and strain B13. These results indicate that 3CB-1 and 3CB-2 are identical but differ from the injected bacterium *Pseudomonas* sp. strain B13. 3CB-1 and 3CB-2 are probably siblings since they were isolated from the same enrichment flask (Thiem *et al.* 1994).

To further establish their phylogenetic relationships, the partial 16S rRNA gene sequences from the novel isolate and strain B13 were analysed with those of the *Proteobacteria* in the RDP and GenBank. The phylogenetic tree constructed by the maximum likelihood method showed that 3CB-1 and 3CB-2 were most closely related to *Alcaligenes eutrophus* in the beta subclass of the *Proteobacteria*, with a sequence similarity of 99.6%, whereas B13 is clustered with *Pseudomonas mendocina* and *P. aeruginosa* in the gamma subclass of the *Proteobacteria*, with sequence similarities of 93.6–94.6% (Fig. 2). Similar tree topologies were obtained using the Neighbour-Joining distance matrix method and bootstrap maximum parsimony methods (data not shown). These results indicate that 3CB-1 and 3CB-2 are phylogenetically quite different from the injected bacterium, *Pseudomonas* sp. strain B13.

The partial sequences of the *clc*D gene from 3CB-1, 3CB-2 and strain B13 were identical within the sequenced 471-bp region. The number of nucleotide substitutions for the

16S genes of these two isolates and B13 was 0.209 ± 0.023 substitutions per nucleotide, whereas the number of nucleotide substitutions for the *clc*D gene were zero between strain B13 and these two isolates. These results suggest that the 3-chlorobenzoate-degrading genes were transferred from B13 to 3CB-1 and 3CB-2.

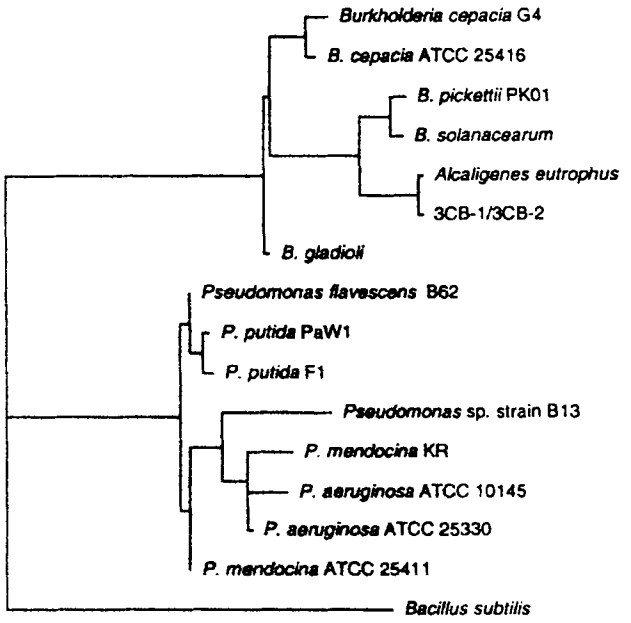

Fig. 2 The maximum-likelihood topology for strain B13, 3CB-1 and 3CB-2 and other related bacteria analysed by the jumble option based on 10 orderings of the data (log likelihood = – 2259). The relative distances between nodes and tips and between internodes are displayed.

To determine whether the whole operon was transferred or only the *clc*D gene was transferred, another gene in the *clc*D gene operon, the *clc*A gene coding for chlorocatechol oxygenase, which is located 3 kb away from *clc*D gene (Frantz *et al.* 1987), was also sequenced. Identical sequences were observed among 3CB-1, 3CB-2 and B13. This suggested that the whole operon was transferred from B13 to these new isolates.

To investigate the possible mechanisms involved in the transfer process, we determined whether the catabolic genes could be found on a plasmid. Only a single DNA band, which corresponded to chromosomal DNA, was observed in strain B13 and these two isolates, whereas two bands were observed for the positive reference strains which contain plasmids (Fig. 3a). This result suggests that no conventional plasmid exists in strain B13 nor in these two isolates. Southern blot analysis showed that the band corresponding to chromosomal DNA did hybridize with *clc*D gene probes (Fig. 3b), indicating that the 3-chlorobenzoate-degrading genes in this novel isolate and strain B13 were either chromosomally encoded or in large DNA fragments associated with the chromosome.

Discussion

The REP-PCR patterns and 16S gene sequence similarity indicated that the two 3-chlorobenzoate-degrading isolates were identical but different from B13. These observations agreed with previous data showing that these two isolates had identical 23S RFLP patterns, but differed from those of B13, and had no hybridization with the B13-specific gene probe (Thiem *et al.* 1994).

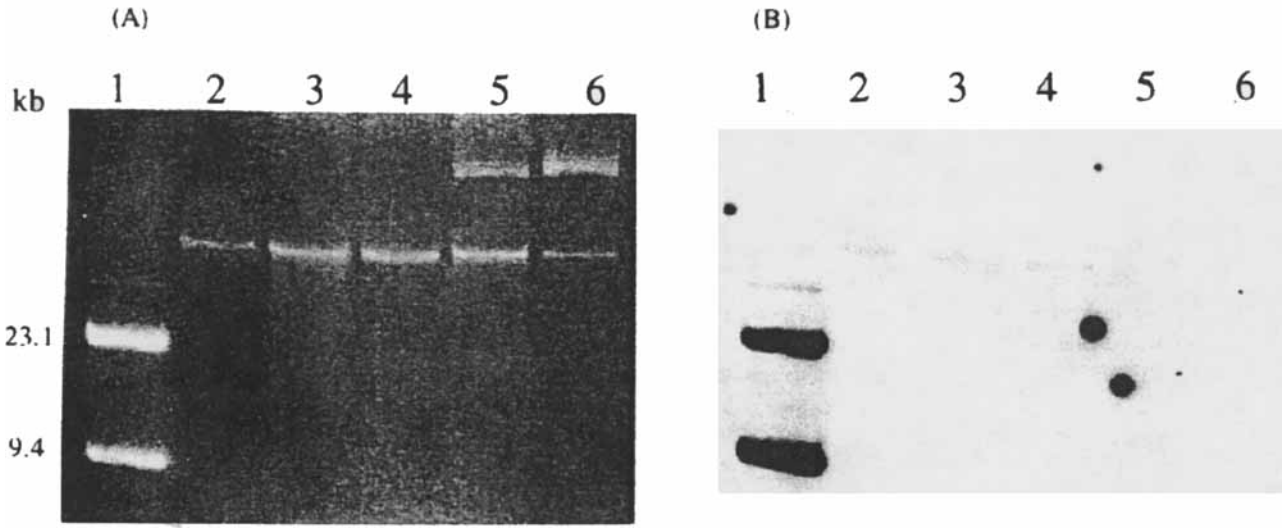

Fig. 3 Plasmid analysis of *Pseudomonas* sp. strain B13 and these two novel isolates. (A) Ethidium bromide-stained gel of plasmid and chromosomal DNA. Lane 1, *Bam*HI-, *Eco*RI- and *Hin*dIII-digested lambda DNA standard; Lane 2, *Pseudomonas* sp. strain B13; Lane 3, 3CB-1; Lane 4, 3CB-2; Lane 5, *Azoarcus tolulyticus* Td-3; Lane 6, Strain 2811p. These two reference strains contain plasmids (Fries *et al.* 1994; Ka *et al.* 1994). (B) Southern blot of the same gel hybridized with the 615 bp fragment of *clc*D genes from *Pseudomonas* sp. strain B13.

The published *clc*D gene sequences from *Pseudomonas putida* and *Pseudomonas* sp. B13 were identical (Frantz & Chakrabarty 1987; Frantz *et al.* 1987). However, a three base difference was observed between the *clc*D gene sequences which we obtained from strain B13 and the published *clc*D gene sequence. In nucleotide positions from 235 to 237, the nucleotide sequence was GCG (Ala) in our sequences instead of CGC (Arg) in the published *clc*D gene sequence from the strain B13. This difference could be real or could be due to sequencing error. We sequenced this region six times from both directions. All of our sequencing results confirmed the 3bp difference we report.

The sequence analyses showed that the novel isolates and strain B13 belong to different subclasses of the *Proteobacteria* but have no sequence divergence for the catabolic *clc*D and *clc*A gene. Since the nucleotide substitution rate of 16S rRNA genes between this novel isolate and B13 was significantly higher than those of *clc*D and *clc*A genes, the 3-chlorobenzoate-degrading genes must have been transferred from *Pseudomonas* sp. strain B13 to these novel isolates.

While no plasmid was detected in the strain B13 that we used, plasmids were observed in the positive reference strains, indicating that the protocol we used was effective for detecting at least conventional plasmids. Also, Southern hybridization showed that the 3-chlorobenzoate-degrading genes were associated with the chromosomal band and maybe chromosomally encoded. This is contrary to the previous observation that the 3-chlorobenzoate-degrading genes were plasmid-borne in *Pseudomonas* sp. strain B13 (Frantz *et al.* 1987; Chatterjee *et al.* 1983). The most likely explanation for this difference is that the 3-chlorobenzoate-degrading genes could now be integrated into the chromosome with subsequent loss of the original plasmid contributing to 3-chlorobenzoate degradation in the B13 clone we used. Such shifts in gene location appear to be common for catabolic traits (Ka & Tiedje 1994; Wyndham *et al.* 1994). Catabolic transposons, which are well documented for another 3-chlorobenzoate-degradation pathway (Fulthrope & Wyndham 1992), could explain the events seen in this study. Since the reported plasmid in the strain B13 was large, about 110 kb (Chatterjee & Chakrabarty 1983), an alternative explanation is that this large plasmid DNA behaved like chromosomal DNA under the isolation conditions used.

While these data show that transfer of the *clc*A and *clc*D genes must have occurred, it is not clear when the transfer occurred. It could have occurred from B13 to the indigenous aquifer strains during the enrichment and isolation processes or during the 14.5 months after B13 injection into the aquifer. The transfer could also have occurred many years prior to the injection from a common ancestral operon. While this historical transfer can not be ruled out, its probability is much lower than the contemporary transfer because no 3-chlorobenzoate degraders could be enriched from the aquifer prior to B13 injection, and even if present at some low level, mutation might be expected in sequence of the two genes, especially in a strain physically isolated (8 m below the surface on Cape Cod, MA) from any known or projected common source. This study illustrates the dilemma in evaluating gene transfer in field studies because the interpretations must usually be based on probability. It is more difficult to argue whether the transfer was *in situ* or *ex situ*, but even if the latter were the case, the work demonstrates that a suitable recipient and transfer mechanism existed in the same local niche (10-g aquifer sample). Hence *ex situ* transfer would suggest a reasonable probability for *in situ* transfer.

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This paper derives from a previous collaborative field study on an aquifer on Cape Cod, MA (cf. Krumme *et al.* 1994). The cooperators included Mary Lou Krumme and Daryl Dwyer of the German Biotechnology Institute, Richard L. Smith and the drilling crews of the U.S. Geological Survey, Suzanne M. Thiem and James M. Tiedje of Michigan State University, the Otis Air National Guard Base officials who granted permission and use of on-site facilities and the US Department of Energy who provided much of the project funding. Particularly important to the current study were the efforts of Drs Krumme, who led the field work, and Thiem who isolated novel strains 3CB-1 and 3CB-2. Dr Zhou is a postdoctorate with background in theoretical ecology and molecular phylogeny. Field studies are expensive, require a wide range of expertise and facilities but are very important in providing ground truth in microbial ecology. This study was an attempt to further maximize knowledge gained from such a field study.
