Microcosms containing sterile soil were used to study the effects of naphthalene, competition from another bacterium, and exchange of the naphthalene-degradative NAH7 plasmid, on the fitness of strains of the gram-negative bacterium *Pseudomonas putida*. Naphthalene had a negative effect on fitness: densities of all three strains tested (naphthalene degrader, isogenic nondegrader, nonisogenic nondegrader) were lower when naphthalene was present in the soil. The NAH7 plasmid provided a selective advantage to the naphthalene degrader relative to its isogenic nondegrader counterpart when the two strains were grown in soil containing naphthalene. The plasmid was disadvantageous in the absence of naphthalene, although both strains persisted under all experimental treatments. In contrast, the relative fitness of nonisogenic strains grown together was controlled by competitive effects: densities of the naphthalene degrader were depressed, and those of the nonisogenic nondegrader elevated, when the two strains were grown together regardless of whether or not naphthalene was present. The rate of horizontal transmission of the NAH7 plasmid was a function of the product of the density of the donor and recipient cells. Vertical transmission of the plasmid as the bacterial cells divided was estimated to account for almost all transconjugants produced after the third day of the experiment. Paradoxically, the greatest development of a transconjugant population occurred in microcosms that did not contain naphthalene, and hence it was not directly due to a selective advantage of naphthalene degradation. Neither selection in the presence of naphthalene nor a long prior period of coevolution was required for stable carriage of the plasmid. The stability of the plasmid in transconjugants, and the high relative fitness of the transconjugants, suggest horizontal transfer is an important component of the evolutionary success of this plasmid. Repeated bouts of horizontal transfer followed by intense competition in soil microcosms may facilitate the selection of strains well suited for bioremediation.

**Introduction**

Plasmids provide opportunities for experimental studies of the evolution of relationships between these extrachromosomal genetic elements and their host bacterial cells. The fitnesses of plasmids and their hosts are intertwined, and there are conflicting ideas about the general or typical nature of this association. Modeling has suggested the relationship may range from mutualistic to parasitic (Stewart and Levin 1977; Levin and Lenski 1983). If mutualistic, the plasmid provides genes for functions such as antibiotic resistance, use of specific carbon sources, and so forth, that are beneficial to the host. However, these functions may be unnecessary under some ecological circumstances, thus making the plasmid a potential burden to its host (Levin et al. 1979; Campbell 1981; Levin and Lenski 1983; Nguyen et al. 1989). A purely parasitic state can be maintained by infectious transmission if host density and the transmission rate of the plasmid are high enough (Stewart and Levin 1977), though these conditions may not be common in nature (Levin et al. 1979).

Genes for degradative pathways are frequently found on plasmids (Frantz and Chakrabarty 1986), and such plasmids can certainly benefit their hosts. It is not clear whether evolution has reduced the cost to the host when selection for functions carried by the plasmid is absent. Studies on the fitness of plasmid-bearing bacterial hosts under nonselective conditions have given conflicting results (Jain et al. 1987; Nguyen et al. 1989; van Overbeek et al. 1990).

The bacterium *Pseudomonas putida* carrying the conjugal degradative plasmid NAH7 (Dunn and Gunsalus 1973; Schell 1990) was used in the current study. NAH7 has the genes for the first 13 enzymatic steps of the naphthalene-degradation pathway and allows its host to use naphthalene as its sole source of carbon.
However, naphthalene is representative of lipophilic aromatic hydrocarbons, found at some contamined sites, which induce toxicity by interfering with cell membrane functions (Yen and Serdar 1988). Naphthalene either could serve as a carbon source for hosts of NAH7, thus aiding their growth, or it could act strictly as a toxic chemical. NAH7 is a large plasmid (83 kb; Schell 1990), and hence it might be expected to be costly to maintain in the absence of naphthalene. However, the naphthalene degradation genes are inducible, which will reduce fitness less than if all the genes were expressed constitutively, and is maintained at a low copy number (Schell 1983).

In this study, the relative fitnesses of a plasmid-bearing degradative microbe, its isogenic plasmidless counterpart, and a third genetically distinct strain (“nonisogenic”), were determined in microcosms containing sterilized soil. The microcosms varied in whether or not naphthalene crystals were added and whether or not only a single strain was present (introduces a potential competitor and/or alternate plasmid host). Microcosms allow control over the amount of biological diversity present and the presence of conditions selective or nonselective for a plasmid function, therefore providing an ecological setting intermediate between the simple one of single strains in broth culture or chemostat and the full complexity of the natural world (Liang et al. 1982; Walter et al. 1991).

Four questions were asked: (1) Does the plasmid confer a net advantage to its host when grown in soil containing naphthalene? (2) Is there a disadvantage to carrying the plasmid when growing in soil without naphthalene? (3) Does growth together with another strain alter the relative fitness of the plasmid-bearing bacteria? (4) Is the fitness of the plasmid-bearing host affected more if the competing strain is isogenic or if it is genetically distinct?

Material and Methods

Strains

First, N+ (*Pseudomonas putida* ATCC 17485, PpG7, kindly provided by Dr. Betty H. Olson, University of California, Irvine), contains the NAH7 plasmid and is sensitive to streptomycin and rifampicin (Sigma Chemical Company, St. Louis, Mo.). NAH7 was originally isolated from this strain (Dunn and Gunsalus 1973). Second, N− is a spontaneous mutant of N+ unable to use naphthalene as a sole carbon source. Third, R is a spontaneous mutant of *P. putida* ATCC 17453 (PpG1) resistant to streptomycin (500 g/ml) and rifampicin (100 g/ml). R does not contain NAH7 or degrade naphthalene. NAH7 readily transfers from PpG7 to PpG1, and transconjugants can use naphthalene as a sole source of carbon (Dunn and Gunsalus 1973).

Media

Total counts were made on L agar (Lennox 1955), a complete medium which supports the growth of all three strains above. N+ cells were identified by growth on MIN plates (minimal medium of Saylor et al. 1985, modified by substituting 5 mg FeCl₃ · 6H₂O for 1.1 mg FeSO₄ · 7H₂O, per liter), with naphthalene crystals added to the lid of each plate. Naphthalene vapors provide the sole source of carbon in MIN plates. Antibiotic resistance was determined using L or MIN plates containing either 500 (g/ml streptomycin sulfate (Lstr, MINstr) or 100 (g/ml of rifampicin (Lrifm, MINrifm).

Soil Microcosms

Each microcosm consisted of 25 g (dry weight) of a premixed commercial potting soil (Bandini® Potting Soil, Bandini Fertilizer Company, Los Angeles, CA 90023) in a peat pot (2” diameter), inside a Pyrex storage dish (4” x 3 ¼”) covered with a glass lid. The greater size of the storage dish relative to the peat pot meant aerobic conditions were maintained over much of the soil volume. The potting soil was composed of finely ground fir bark, Canadian sphagnum peat moss, composted redwood bark, and sterile horticultural sand. Soil for all the microcosms was taken from the same well-homogenized bag. The soil was moistened with 50 ml deionized water, then the entire microcosm sterilized by autoclaving for 90 min. The soil physical and chemical characteristics after autoclaving were determined by the Soil, Water and Plant Analysis Laboratory of the University of Arizona, Tucson, using standard techniques: sand (85%), silt (11.8%), clay (3.2%), pH 4.60, electrical conductivity (9.84 mmhos/cm), total nitrogen (0.38%), organic carbon (5.05%), and soluble forms of calcium (1380 μg/g), sodium (99.6 μg/g), potassium (333 μg/g), magnesium (390 μg/g), nitrate (1240 μg/g), and phosphate (226 μg/g). Following autoclaving, 1 g of naphthalene (found upon testing to be sterile) was added to 13 of the microcosms (see table 1), mixed thoroughly with the soil to ensure an even distribution, and incubated for 3 d to allow the naphthalene to equilibrate before inoculation with the bacteria. Part of the naphthalene would be accessible to bacteria in the aqueous layer (solubility of 31.7 mg/l) or as vapor in the air (0.23 mm Hg), and a portion might be less available due to sorption to soil particles (Javert 1991). Where added, naphthalene crystals were still visible at the end of the experiment.

Experimental Design

Two sets of microcosms were set up as described in table 1. The initial inoculation was made from frozen stocks of the strains which were thawed, diluted into a sterile inorganic salts solution (Duggleby et al. 1977),
Table 1
Experimental Design

<table>
<thead>
<tr>
<th></th>
<th>Isogenic</th>
<th></th>
<th>Not Isogenic</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Strain 1 alone</td>
<td>N+ (2*)</td>
<td>N+ (2)</td>
<td>N+ (2)</td>
<td>N+ (2)</td>
</tr>
<tr>
<td>Strain 2 alone</td>
<td>N− (2)</td>
<td>N− (2)</td>
<td>R (2)</td>
<td>R (2)</td>
</tr>
<tr>
<td>Strains 1, 2 together</td>
<td>N+, N− (2)</td>
<td>N+, N− (2)</td>
<td>N+, R (3)</td>
<td>N+, R (3)</td>
</tr>
<tr>
<td>Total no. of microcosms</td>
<td>12</td>
<td>...</td>
<td>16**</td>
<td>...</td>
</tr>
</tbody>
</table>

Note.—Treatments: Strain 1 grown singly, strain 2 grown singly, strains 1 and 2 grown together, with ("Yes") or without ("No") 1 g of naphthalene in the soil microcosm. A single asterisk (*) indicates number of replicate microcosms, two asterisks (**) means number also includes two cultures without bacteria for sterility controls. Microcosms were sampled as described in Material and Methods on the following schedule (numbers refer to days after initial inoculation): 1, Isogenic, 2, 5, 9, 16, 22; and 2, Not Isogenic, 1, 2, 3, 7, 10, 14, 21.

then immediately added to the microcosms. Initial densities, estimated as cfu's/g soil after dilution from the stock and plating on L agar, were Isogenic (N+ = 7.7 × 10^5, N− = 8.8 × 10^5), Not Isogenic (N+ = 1.74 × 10^6, R = 1.69 × 10^6).

Treatment and Sampling

Incubation was at room temperature (23°−25°C), with sterile water added as needed to the bottom of the glass container to be wicked up by the peat pot to keep the soil moist, but not saturated. Percent water in the soil (wet mass) was determined to be 63% by oven-drying samples to a constant weight. All microcosms were kept in Rubbermaid® plastic containers to preserve constant levels of soil moisture. Microcosms with and without naphthalene were kept in separate incubators. One-gram soil samples, pooled from three separate samples taken from the surface down to 1 cm below the surface, were removed from each microcosm with a sterile spatula at intervals over a period of 22 d (table 1), vortexed at highest speed together with 5 ml of sterile buffer, diluted, plated, and incubated at room temperature. Two microcosms set up as sterile controls were sampled in parallel with experimental cultures; they remained sterile throughout the duration of the experiment, thus demonstrating the efficacy of the sterilization procedure.

Identification of Colonies

Single colonies were transferred with sterile toothpicks from spread plates onto the various types of media to determine their phenotype. Transconjugants (N+ donor, R recipient) were initially selected on MINrfm plates. Rifampicin-resistant mutants of N+, identified initially by their N+ -like colony morphology (N+ and R differ in colony morphology) on MINrfm, were further distinguished from transconjugants by sensitivity to streptomycin. The average frequency of mutation of N+ to rifampicin resistance was estimated to be 8.32 × 10^-8 (calculated from microcosms where N+ was grown alone).

Stability of NA7 Plasmid in Transconjugants

Transconjugants isolated from MINrfm plates (Not Isogenic experiment) were tested for retention of NA7 as follows: (1) five transconjugant colonies from each two-strain microcosm were diluted and spread onto nonselective L plates, then (2) 43–50 single colonies from each L plate were transferred to MINrfm plates to test for growth. Colonies tested were from two different sampling times: an early sample taken on days 1–3 from two microcosms with naphthalene (group 1, see table 3) and two microcosms without naphthalene (group 2); and a late sample taken on day 21 from three microcosms with naphthalene (group 3) and three microcosms without naphthalene (group 4).

Statistical Tests

Relative fitness was estimated by calculating the selection coefficient (SC) as the slope of a least-squares linear regression of the natural logarithm of the ratio of the density of one strain to the density of a second strain versus time, in days (Dykhuizen and Hartl 1983; Lenski 1992). Student’s t-test for estimating the SC (table 2) and comparing two SCs was calculated using GraphPad InStat® (Motulsky 1993). Student’s t-test for comparing two slopes and comparing two elevations (Y intercept of a line) was taken from Zar (1984).

Plasmid transfer rates (γ) were estimated as

\[ γ = ψ/(N_0 − N_o) \cdot \ln \{ [R + ρ(b)] / [R + ρ(a)] \} \]  

(eq. [5] from Levin et al. 1979); where \( R = n+/N \), \( n+ = \) density of donors, \( N = \) total density, \( ρ(b), ρ(a) = n−/n \) at times \( b \) and \( a \) during exponential phase, \( n− = \) density of transconjugants, \( n = \) density of recipients, \( ψ = \) the growth rate during the exponential phase, and
Table 2

Selection Coefficients (SC)

<table>
<thead>
<tr>
<th></th>
<th>SC</th>
<th>SEM*</th>
<th>t^b</th>
<th>df</th>
<th>P^p</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Strains grown separately:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isogenic:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naphthalene+</td>
<td>−0.086</td>
<td>0.0237</td>
<td>3.629</td>
<td>5</td>
<td>0.0075*</td>
</tr>
<tr>
<td>No naphthalene:</td>
<td>+0.070</td>
<td>0.0409</td>
<td>1.711</td>
<td>5</td>
<td>0.0738</td>
</tr>
<tr>
<td>Not Isogenic:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naphthalene+</td>
<td>−0.092</td>
<td>0.0544</td>
<td>1.691</td>
<td>7</td>
<td>0.0673</td>
</tr>
<tr>
<td>No naphthalene</td>
<td>−0.016</td>
<td>0.0439</td>
<td>0.365</td>
<td>7</td>
<td>0.3631</td>
</tr>
<tr>
<td>II. Strains grown together:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isogenic:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naphthalene+</td>
<td>−0.101</td>
<td>0.0589</td>
<td>1.715</td>
<td>4</td>
<td>0.0808</td>
</tr>
<tr>
<td>No naphthalene:</td>
<td>+0.060</td>
<td>0.0434</td>
<td>1.382</td>
<td>4</td>
<td>0.1195</td>
</tr>
<tr>
<td>Not Isogenic:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naphthalene+</td>
<td>+0.118</td>
<td>0.0348</td>
<td>3.391</td>
<td>7</td>
<td>0.0050*</td>
</tr>
<tr>
<td>No naphthalene</td>
<td>+0.059</td>
<td>0.0345</td>
<td>1.710</td>
<td>7</td>
<td>0.0655</td>
</tr>
</tbody>
</table>

NOTE.—Selection coefficients were estimated from the slope of the regression line of the ratio of ln densities (N−/N+) or (R/N+) vs. time, in days (Lenski 1992).

* SEM, standard error of the mean for the SC (slope).

^b t, Student’s t-test, null hypotheses: Is slope < 0 when grown in soil with naphthalene? Is slope > 0 when grown without naphthalene? Naphthalene+, grown in soil with naphthalene. No naphthalene, grown in soil without naphthalene.

\[ \gamma = \psi \ln(1 + T/R \cdot N/D) \cdot 1/[N−N_0] \]  
(2)

(end-point estimation model; Simonsen et al. 1990),

where \( \psi \) = the growth rate during the exponential phase, \( T \) = density of transconjugants, \( R \) = density of recipients, \( N \) = total density, \( D \) = density of donors, and \( N_0 \) = initial total density.

Plasmid stability data were analyzed with the ONEWAY and ANOVA procedures (SPSS/PC+; Norusis 1988). Each of the four groups in table 3 was tested to determine whether the microcosms in each group formed a homogeneous set of data. Group 3 had a significant between-group component (\( F = 9.4466, df = 2.14, P = 0.0034 \)); therefore, these data were eliminated from the pooled analysis.

Results

Fitness of Plasmid and Hosts under Various Treatments

Effect of Naphthalene

Naphthalene was somewhat toxic to all three strains: all three strains reached higher densities in soil without naphthalene than they did with naphthalene (fig. 1).

Relative Fitness (SC)

Figure 2 (left-hand side) shows the relative fitness of N− or R compared to that of N+ when grown separately versus when grown together (right-hand side). The significantly negative SC (table 2, section 1) shows that N− does not maintain as high a density as N+ does in the presence of naphthalene, suggesting that NAH7 detoxifies naphthalene for its host and/or allows naphthalene to be used as a carbon source. In both experiments the SC is more negative for growth with than without naphthalene, but only significantly different in the isogenic experiment (SC: naphthalene present = −0.086 vs. no naphthalene = +0.070; unpaired t-test, \( t_{10} = 3.30, P_{(2)} = 0.0079 \)).

When the two isogenic strains were grown together, SC had the same sign and was close to the same value as when the isogenic strains were grown alone. N− appeared to do slightly worse when grown together with N+ than when grown separately (naphthalene present: −0.086 vs. −0.101, no naphthalene: +0.070 vs. +0.060). This small difference may reflect the conversion of N− to N+ cells by transfer of the plasmid; except for the plasmid, the strains are otherwise indistinguishable. However, both the sign and the relative magnitude of the SC changed when the R and N+ pair were grown together rather than separately. R did much better than N+ when they were grown together and worse when grown separately in the presence of naphthalene (SC:

Table 3

<table>
<thead>
<tr>
<th>Plasmid Stability in Transconjugants</th>
<th>Total Number</th>
<th>Mean % Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tested</td>
<td>(SEM)</td>
</tr>
<tr>
<td>Group 1: Early sample (days 1–3), naphthalene present:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microcosm 1</td>
<td>250</td>
<td>95.6 (0.98)</td>
</tr>
<tr>
<td>Microcosm 3</td>
<td>250</td>
<td>89.2 (2.87)</td>
</tr>
<tr>
<td>Group 2: Early sample (days 1–3), naphthalene absent:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microcosm 1</td>
<td>250</td>
<td>89.6 (4.53)</td>
</tr>
<tr>
<td>Microcosm 2</td>
<td>250</td>
<td>97.2 (1.50)</td>
</tr>
<tr>
<td>Group 3: Late sample (day 21), naphthalene present:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microcosm 1</td>
<td>250</td>
<td>92.8 (1.62)</td>
</tr>
<tr>
<td>Microcosm 2</td>
<td>243*</td>
<td>80.3 (4.16)</td>
</tr>
<tr>
<td>Microcosm 3</td>
<td>250</td>
<td>96 (1.41)</td>
</tr>
<tr>
<td>Group 4: Late sample (day 21), naphthalene absent:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microcosm 1</td>
<td>250</td>
<td>100 (0.00)</td>
</tr>
<tr>
<td>Microcosm 2</td>
<td>250</td>
<td>99.2 (0.49)</td>
</tr>
<tr>
<td>Microcosm 3</td>
<td>250</td>
<td>100 (0.00)</td>
</tr>
</tbody>
</table>

NOTE.—Five transconjugant colonies from each microcosm (Not isogenic experiment) were separately diluted and plated on nonselective media; then 50 colonies derived from each transconjugant colony (total number tested) were transferred and scored for growth (Mean % growth) on selective MInEm plates. SEM, one standard error of the mean. An asterisk (*) indicates that 43 colonies were tested from one transconjugant.
together = +0.118, vs. alone = −0.092; unpaired t-test, 
$t_{14} = 3.391, P(2) = 0.0058$).

Change in the Percentage of Plasmid-Bearing Cells

In the Isogenic experiment, the percentage of N+ cells increased from their initial levels in microcosms containing naphthalene and decreased in those without naphthalene. Both N+ and N− persisted in all microcosms, and their numbers relative to each other did not appear to reach equilibrium during the 3 wk of the experiment. In contrast, when N+ and R were together, N+ decreased in the presence or absence of naphthalene after a short initial period of increase. The percentage of naphthalene-degrading cells increased during the latter part of the experiment in microcosms without naphthalene due to the rise of R+ (transconjugant cells).

Conjugation and Transfer of the NAH7 Plasmid (Not Isogenic)

Transconjugants Were Produced in the Soil, Not on Selective Plates

The number of transconjugants in the soil will be overestimated if conjugation occurs on the selective media used for sampling (Smit and van Elsas 1990). Conjugation on plates was tested by spreading together on a MINrfrm plate $5.25 \times 10^6$ cfu's each of N+ and R cells grown separately in broth. No transconjugants were produced. The tested densities produced 75–100 transconjugants per plate on a medium which permitted conjugation (mineral salts medium of Chakrabarty et al. 1973, containing 500 μg/ml of streptomycin).

Dynamics of Transconjugant Formation

Transconjugants were found in all six soil microcosms in which N+ and R were grown together (fig. 3). No colonies with the transconjugant phenotype appeared in any of the single-strain, control microcosms, despite repeated sampling. Transconjugants from soil with naphthalene formed at most only a small fraction of the population (e.g., 0.11% of the total on day 21). A striking increase in R+ (transconjugants) occurred in microcosms without naphthalene. On day 21 R+ comprised 28.5% of the entire population, slightly higher than that of N+ (25.9%).

Rates of Plasmid Transfer

Horizontal transmission of plasmids (conjugation) is expected to predominate when donor cells first contact recipient cells. The number of transconjugants produced initially in a homogeneous, well-mixed liquid environment will be largely a function of the product of the density of the donor and recipient cells (a mass-action process).

![Graph of Strains grown alone](image)

**FIG. 1.**—Time course of total population densities of N+, N−, and R alone in microcosms with and without naphthalene crystals added to the soil. Each point, in terms of log_{10} colony-forming units per gram of soil (wet weight), is the average of two soil microcosms, except where an asterisk (*) indicates that only one microcosm was sampled on that date. Solid and open symbols designate microcosms with and without naphthalene, respectively. Triangles indicate N+; circles, N− or R. Error bars are plus and minus 1 SE of the mean. Some error bars are too small to be visible at this scale. Top, Isogenic experiment; bottom, Not Isogenic experiment.

Mass-Action Effect of Cell Densities

When the density of transconjugants was plotted as a function of the product of the donor (N+ and R+) and recipient (R) densities present during the first 3 d (log_{10} transconjugant cells/g soil vs. log_{10} donor cells/g soil × log_{10} recipient cells/g soil, for days 1–3), the points describing the density of newly arising R+ transconjugants fell along a straight line, as predicted by a mass-action model. R+ densities from only the first 3 d were used to lessen the confounding effects of increase by vertical transmission, although some R+ cells that arose from binary fission of preexisting R+ cells were undoubtedly included. A first-order regression line plotted through the points for the microcosms without naphthalene had slope $= 5.45 \times 10^{-14}$ and $Y$ intercept $= -123.4 (r^2 = 0.68)$, those with naphthalene had slope $= 1.94 \times 10^{-14}$ and $Y$ intercept $= -35.3 (r^2 = 0.85)$. The slopes were not significantly different from each
other, nor were the Y intercepts (t-test, P > 0.50), indicating that naphthalene did not change the mass-action relationship. Rather, fewer plasmid transfers are expected due to the lower donor and recipient densities in microcosms with naphthalene.

**Estimating Rates of Transfer**

The plasmid transfer rate (γ) from the mass-action model (MA) of Levin et al. (1979) was calculated to compare horizontal transfer frequencies in uncontaminated soil and contaminated soil while allowing for lower parental densities in the microcosms containing naphthalene. Although the MA model was developed for cells in liquid culture, the mass-action relationship described above justifies use of this model, even though it is not likely that conditions in the soil microcosms meet all the assumptions of the model. γ (transfers in 1 g soil cell⁻¹ hr⁻¹) was 1.7 × 10⁻¹⁵ in microcosms with naphthalene and 3.1 × 10⁻¹⁵ in microcosms without naphthalene, using cell densities and growth rates estimated during the period from inoculation to day 1. These rates do not differ significantly (t(3) = 0.8563, P(2) = 0.4548). The dashed line describes the number of transconjugants expected using the γ values. The number in microcosms with naphthalene is overestimated early in the experiment, then levels off at a density below the observed. The fit is better between the observed and estimated before day 5 in microcosms without naphthalene, but the estimated densities are much below the observed during the last 2 wk of the experiment. γ values are expected to be at their maximum during exponential growth (Levin et al. 1979), and those calculated after day 1 were lower (average for days 2–21: 7.2 × 10⁻¹⁸ with naphthalene, 1.5 × 10⁻¹⁸ no naphthalene).

The end-point estimation model (EP; Simonsen et al. 1990) was specifically developed for batch cultures, and hence it may be better suited to the present study. γ values calculated using densities at inoculation and at day 1 were 1.8 × 10⁻¹⁶ g soil cells⁻¹ hr⁻¹ for microcosms with naphthalene and 2.8 × 10⁻¹⁵ for those without. These rates were not significantly different from each other (t(3) = 2.596, P(2) = 0.0806) or from the MA rates above (all t values < 3.863, P > 0.05, Bonferroni multiple-comparisons test). The dotted line is the number of transconjugants expected using the γ values. Again, the number of transconjugants in microcosms with naphthalene was overestimated early in the experiment, then leveled off at a density well below that observed during the last week. The fit was excellent between ob-
served and estimated numbers before day 5 in microcosms without naphthalene, but the estimated densities were again well below the observed during the last 2 wk of the experiment.

**Vertical versus Horizontal Plasmid Transmission**

The transconjugants estimated to be contributed by horizontal transfer quickly hit a plateau, due to a decline in total cell density as the experiment progressed (fig. 3), thereafter binary fission (e.g., vertical transfer) of preexisting transconjugants was responsible for most of the succeeding rise in R+. An increase in R+ while both N+ and R were declining implies that R+ had the higher relative fitness.

**Stability of the NAH7 Plasmid in Transconjugant Cells**

Transconjugants were tested to determine how frequently NAH7 was lost after a short period of growth in a medium without naphthalene. Eighty to 100% of R+ colonies transferred from selective MINrfm medium and tested after a short period of growth on nonselective L agar retained the ability to grow on naphthalene (table 3). More naphthalene-degrading colonies were found in samples taken at later times (two-way ANOVA, \( F_1 = 8.485, P = 0.006 \)) and from microcosms without naphthalene (\( F_1 = 5.041, P = 0.03 \)). Although inability to grow on naphthalene does not test directly for the loss of NAH7, the 0.3%–7.6% of colonies unable to grow on naphthalene is consistent with previous estimates of rates of plasmid loss (Lenski and Bouma 1987) and far greater than would be expected for single-base mutations of naphthalene degradation genes.

**Discussion**

Our results demonstrate significant fitness consequences for a degradative plasmid (NAH7) and its host bacterium (*Pseudomonas putida*) when placed in a microcosm incorporating certain minimal forms of ecological realism: (1) genetic complexity: interactions among bacteria of different genetic backgrounds, (2) evolutionary complexity: formation of a new plasmid-host association, and (3) physical complexity: soil carbon sources in addition to the selective agent. In each treatment, the number of cells increased 100- to 1,000-fold, or approximately 6–8 generations (calculated from estimates of doubling times) during the first 2 d of the experiment but thereafter remained nearly stationary or declined during the succeeding 19 d. Relative fitness in this study primarily measured the ability to persist in an environment as resources dwindled. Due to spatial and temporal variation in the level of nutrients, microbes in the natural soil environment also may experience long periods of nutrient limitation interspersed with only short bursts of cell division (Williams 1985).

**Genetic Complexity**

The fitness measures reflected the strong influence of interactions among strains. However, the persistence of N+ cells in the face of competition from an isogenic strain under nonselective conditions indicates that the disadvantage of carrying NAH7 was relatively minor (table 2: No naphthalene—the SC was not significantly different from zero).

**Possible Basis of the Competition: Diversion of Degradation Products?**

Although R survived quite well alone in these soil microcosms, R may have gained additional nutrients from N+ (‘‘cross-feeding’’); colonies of R on MIN media were larger, though still very small, when N+ colonies were also present on the plate. Intermediate metabolites have been found in the culture fluid of PpG7 (N+ is PpG7) grown on naphthalene (Abbott and Gledhill 1971). Catechol is a common intermediate in the degradation pathway of aromatic hydrocarbons (Harayama and Timmis 1989), including the catabolism of naphthalene by PpG7 (Dunn and Gunsalus 1973). PpG1, the parent strain of R, has a chromosomally encoded catechol degradation pathway, the β-ketoacidipate pathway (Ornston 1971; Dunn and Gunsalus 1973). The advantage of R over N+ might be one example of a strategy which allows nondegraders to persist in chemically contaminated environments: they benefit from the detoxification efforts of the degraders, and use the extracellular products of the degradative microbes.

**Does Competition Affect the Evolution of Catabolic Operons?**

The need to shield intermediate compounds from competitors may have influenced the evolution of certain biochemical pathways. Many soil bacteria possess the β-ketoacidipate pathway and show chemotactic responses toward some of the compounds in the pathway (Ornston 1971). PpG7 metabolizes the catechol produced during the degradation of naphthalene primarily by the α-ketoacidipate pathway, whose genes are located on NAH7 (Dunn and Gunsalus 1973). The α-ketoacidipate pathway should prevent compounds downstream of catechol from being used by competing bacteria that do not possess that pathway. Thus, the functional redundancy of certain biochemical pathways may reflect a “competitive arms race,” with a selective advantage accruing to the acquisition of alternate pathways leading from a common intermediate to a common central metabolite.
Evolutionary Complexity

Consequences of Plasmid Transfer

In the current study, the interplay between horizontal transfer and vertical transfer of the plasmid was investigated under two conditions: one in which the plasmid was expected to be selectively advantageous, the other in which carrying the plasmid might be disadvantageous. Although a stable association of NAH7 with R was not unexpected (Dunn and Gunsalus 1973), the high relative fitness shown by NAH7 in association with R (R+; fig. 3) had not been demonstrated, nor has a quantitative assessment of the stability of the R+ plasmid-host association (table 3).

The attributes of a plasmid which can spread to many genetically different strains or even to different species are quite different from those of a plasmid whose fate is linked to the persistence of a circumscribed set of hosts. The ability of a plasmid to transfer to and persist in genetic backgrounds different from that of the original host may prove crucial in two ways to the plasmid’s maintenance and spread under nonselective conditions: (1) increased density of hosts makes an infectious transference process more likely; (2) by sampling more hosts the plasmid may have a greater chance of acquiring one on which it has a strongly positive effect on fitness, thus increasing the plasmid’s representation in subsequent generations through vertical transmission. In a practical sense, the degree of independence of degradative plasmids and their hosts is of central importance to the design of procedures for biological remediation.

Plasmid Transfer Rates

The number of transconjugants produced initially resembled a mass-action process. Modifications of soil physical parameters (Richaume et al. 1989) which increase the frequency of plasmid transfer may have as an underlying factor the promotion of high densities of donor and recipient cells. Most studies have not explicitly considered joint parental densities, with some exceptions (van Elsas et al. 1987; Sayre and Miller 1991).

The current study differed in several ways from previous research: use of a large catabolic plasmid, transfer in soil, and rates obtained under both selective and nonselective conditions. Most work on plasmid transfer rates (γ) has been performed for small plasmids of Escherichia coli K12 grown in liquid culture, where rates ranged from $1 \times 10^{-9}$ to $1 \times 10^{-12} \text{ ml cell}^{-1} \text{ hr}^{-1}$ (Levin et al. 1979; Simonsen et al. 1990). Transfer rates between E. coli strains recently isolated from natural populations were much lower (median γ = $5 \times 10^{-16}$ ml cell$^{-1}$ hr$^{-1}$; Gordon 1992). Transfer of TOL (toluene degradation plasmid) in liquid culture among strains of Pseudomonas was estimated as $1 \times 10^{-14}$ to $5 \times 10^{-13}$ ml cell$^{-1}$ min$^{-1}$ (Smets et al. 1993). In one of the few experiments performed in soil, transfer of the recombinant plasmid R388::Tn1721 between strains of P. cepacia was estimated to be $5 \times 10^{-14}$ g soil cell$^{-1}$ hr$^{-1}$ (Knudsen et al. 1988), somewhat higher than the values in the current study. Despite the higher γ, transconjugants reached their highest level within the first 2 d then declined, as would be expected if a mass-action process prevailed initially but the transconjugants produced were not more fit than the parental strains.

Physical Complexity

The inclusion of multiple sources of carbon permitted the microcosms to incorporate an essential feature of microbial life in complex habitats, an ordered sequence of resource use. Bacteria use some carbon sources in preference to others, and the more easily metabolized substrates are often used first (Reber and Kaiser 1981; Schmidt and Alexander 1985).

Naphthalene

The original function of degradative plasmids may have been to detoxify hydrocarbons, not to use them as a source of energy (Farrell and Chakrabarty 1979). Indeed, the toxic properties of naphthalene prevailed in the current experiment (fig. 1). Guerin and Jones (1988) suggested that the quick conversion of hydrophobic aromatic hydrocarbons to water-soluble polar intermediates (Abbott and Gledhill 1971) might act as a form of detoxification. Even though N+ did not eliminate naphthalene from the microcosm, the area immediately surrounding the cell might be detoxified. If so, then N+ would be expected to be less affected than N− or R by naphthalene. Densities of N−, but not of R, were relatively lower than those of N+ in soil with naphthalene. R, derived from PgG1, may possess some ability to detoxify naphthalene (Kulisch and Vilker 1991). Alternatively, R might be able to partially block the uptake of naphthalene: the availability of naphthalene sorbed to soil was quite different for two strains (Guerin and Boyd 1992).

Other Carbon Sources

NAH7 may confer additional advantages to its hosts in soil environments: in soil without naphthalene, R+ grew more rapidly than R, and N+, when grown alone, attained higher densities than did R. Although purely speculative at this point, soil organic matter contains highly complex aromatic chemical compounds (Paul and Clark 1989) whose catabolism or detoxification might be facilitated by NAH7. NAH7 does allow its host to mineralize other polyaromatic hydrocarbons (Sanseverino et al. 1993).
A Practical Side to the Experiments: Implications for Bioremediation

Why do contaminated soils persist despite the presence of naturally occurring microorganisms capable of degrading the contaminating chemicals? Many factors can intervene to decrease degradation rates in nature from those measured for single strains in the laboratory (Liu and Sulfita 1993). Abiotic factors affect rates of degradation and the densities of degradative microbes (Leahy and Colwell 1990; Atlas and Bartha 1992). Competition, predation, and mutualism are also important in determining the fitness of microbes in nature (Alexander 1984).

Our experiments provide clues to solving some problems in bioremediation. If the highly fit counterpart of R+ can be produced as readily as in this study, then one could select for competitively superior plasmid-host combinations through repeated microcosm experiments using the original contaminated soil and its microbes. Ka et al. (1994), in a study of competition among 2,4-D degrading bacteria inoculated into a native soil, found a strain produced experimentally by conjugation outcompeted the rest in both broth and native soil. If we understood the genetic basis of characteristics which determine superior competitiveness, it should be possible to design highly fit plasmid-host combinations.

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