Phenotypic Plasticity in Bacterial Plasmids

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ABSTRACT

Plasmid pB15 was previously shown to evolve increased horizontal (infectious) transfer at the expense of reduced vertical (intergenerational) transfer and vice versa, a key trade-off assumed in theories of parasite virulence. Whereas the models predict that susceptible host abundance should determine which mode of transfer is selectively favored, host density failed to mediate the trade-off in pB15. One possibility is that the plasmid's transfer deviates from the assumption that horizontal spread (conjugation) occurs in direct proportion to cell density. I tested this hypothesis using *Escherichia coli*/pB15 associations in laboratory serial culture. Contrary to most models of plasmid transfer kinetics, my data show that pB15 invades static (nonshaking) bacterial cultures only at intermediate densities. The results can be explained by phenotypic plasticity in traits governing plasmid transfer. As cells become more numerous, the plasmid's conjugative transfer unexpectedly declines, while the trade-off between transmission routes causes vertical transfer to increase. Thus, at intermediate densities the plasmid's horizontal transfer can offset selection against plasmid-bearing cells, but at high densities pB15 conjugates so poorly that it cannot invade. I discuss adaptive *vs.* nonadaptive causes for the phenotypic plasticity, as well as potential mechanisms that may lead to complex transfer dynamics of plasmids in liquid environments.

ALTHOUGH plasmids can provide beneficial traits such as antibiotic resistance, in the absence of positive selection a plasmid typically reduces the growth of its bacterial host *in vitro* (Levin 1980; Zund and Lebek 1980; Lenski and Bouma 1987; Nguyen *et al.* 1989) and *in situ* (Devanas *et al.* 1986; Sundin *et al.* 1994; Bjorklof *et al.* 1995; Moenne-Loccoz and Weaver 1995). Plasmids are vertically transferred to daughter cells during binary fission and can be horizontally transferred from infected cells (donors) to uninfected cells (recipients) through conjugation (Lederberg and Tatum 1946; Clewell 1993; Thomas 2000). Plasmid activities that promote horizontal transmission (such as producing more conjugative pili) should further impede host growth, thereby reducing the plasmid's potential for vertical transmission. We previously demonstrated this trade-off when plasmids evolved increased horizontal transfer at the expense of decreased vertical transfer and vice versa (Turner *et al.* 1998).

Spread of conjugative plasmids in liquid culture is often approximated through simple mass-action models (Stewart and Levin 1977; Levin *et al.* 1979; Bergstrom *et al.* 2000; Ganusov and Brilkov 2002; Paulsson 2002). Matings between donors and recipients are assumed to be governed by random encounters, which result in the creation of plasmid-bearing transconjugants. If matings occur in proportion to donor and recipient concentrations the rate of change of plasmid-bearing cells is

$$dP/dt = \psi_P P + \gamma PR,$$

where P is the combined densities (cells per milliliter) of donors and transconjugants, R is recipient density, ψ_P is exponential growth rate of plasmid-bearing cells, and γ is the rate of conjugative transmission. The per-capita rate,

$$r = dP/Pdt = \psi_P + \gamma R,$$

reveals that vertical spread (ψ_P) is independent of host density, whereas horizontal spread (γR) should be proportional to recipient density.

For parasites that feature both horizontal and vertical modes of transmission, susceptible host density (R) should determine which mode is selectively favored (May and Anderson 1983; Herre 1993; Bull 1994; Ewald 1994), provided there is a genetic trade-off between transfer modes. Consider three hypothetical plasmids: *x*, *y*, and *z*. Plasmid *x* conjugates faster than plasmid *y*, but the trade-off causes slower growth of *x*-infected hosts. Plasmid *z* transfers only vertically, thereby causing the least host burden. The model predicts that *x* and *y* should spread through the host population faster as R increases (Levin *et al.* 1979; Simonsen *et al.* 1990; Figure 1A). In this way, a costly plasmid can theoretically invade a population if its rate of horizontal transfer is rapid enough to offset its poor vertical transmission (Stewart and Levin 1977). At high densities *x* is most favored to invade, whereas the advantage progressively shifts to plasmids *y* and *z* as R decreases. However, any plasmid

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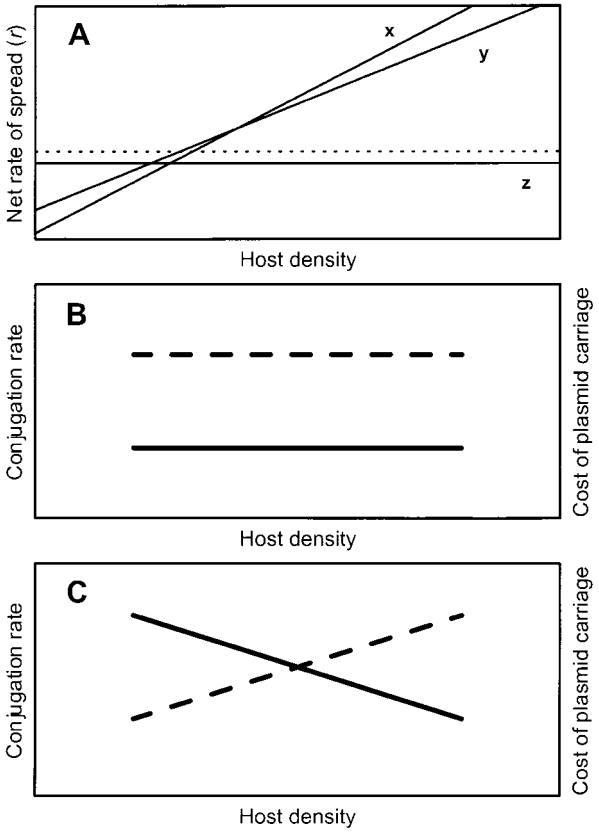

FIGURE 1.—Theorized effect of susceptible host density on plasmid transmission and phenotypic plasticity of plasmid traits. (A) Hypothetical net rates of increase (solid lines) for plasmid genotypes *x*, *y*, and *z*, as a function of available host density. The rapidly conjugating plasmid *x* is favored at high densities, but a trade-off between transmission modes causes the advantage to shift in favor of plasmids *y* and *z* as densities decline. For any plasmid to spread, the net rate must exceed the per-capita growth rate of plasmid-free recipients in the population (dashed line). (B) Reaction norms for two hypothetical plasmids featuring traits unaffected by host density. The solid line designates a plasmid that conjugates slowly but imposes a low cost of carriage, whereas the dashed line indicates a faster-conjugating plasmid that imposes a higher cost. (C) Reaction norms for two hypothetical plasmids featuring phenotypically plastic traits. The solid line shows a plasmid whose conjugation rate and cost of carriage decline with increasing host density, whereas the dashed line symbolizes a plasmid featuring the opposite response. Most models predict that rapid conjugation can be selectively favored at high densities; thus, a correspondingly "optimal" plasmid should feature the latter form of plasticity.

can fail to invade if its net rate of spread is negative ($r < \psi_R - \gamma P$), where ψ_R is the growth rate of recipients (Figure 1A).

Plasmid transfer on surfaces (such as in biofilms) is more complicated than in well-agitated liquid culture, because heterogeneous environments can lead to "patchy" distribution of donors and recipients (SIMONSEN 1990; LICHT *et al.* 1999; LAGIDO *et al.* 2003). However, in either environment increasing cell densities should generally improve plasmid spread (up to a possible saturation point) because the greater cell-to-cell contact affords more opportunities for horizontal transfer. The improvement is generally *not* due to changes in the rate constant of conjugative transfer (γ) or in the cost of plasmid carriage (c), which can be estimated as the difference between recipient and donor growth rates:

$$c = \psi_R - \psi_P.$$

[Cost of carriage can be more broadly defined in terms of the fitness difference between plasmid-free and plasmid-bearing hosts (BOUMA and LENSKI 1988; TURNER *et al.* 1998), whereby growth rate is only one of several fitness components.] Thus, both γ and c are assumed to remain constant as host density varies (Figure 1B). But in the absence of genetic constraints, selection should favor parasites that are phenotypically plastic and able to switch between transmission strategies in direct response to environmental conditions (BRADSHAW 1965; SCHEINER 1993). Therefore, a phenotypically plastic plasmid might be expected to maximize the conjugation rate at high densities to facilitate horizontal spread and to minimize conjugation at low densities to promote vertical spread (Figure 1C).

In a previous study (TURNER *et al.* 1998) we predicted that high host densities should favor evolution of increased plasmid conjugation at the expense of reduced vertical transmission, whereas low densities should favor the reverse. Plasmid pB15 (LUNDQUIST and LEVIN 1986) was allowed to evolve for 500 generations in replicated batch-culture environments containing different availabilities of uninfected *Escherichia coli* hosts. Bacterial concentrations (governed by sugar concentration in the liquid medium) were high ($\sim 10^9$ cells/ml) to facilitate contact between donors and recipients, but differences in *available* host density were achieved by manipulating the immigration of recipients into treatment populations. To prevent disruption of mating pairs the experimental populations were grown in static culture, where matings can also occur between a minority subpopulation of cells at the bottom surface of the culture tube. Results showed that plasmids evolved greater conjugation at the expense of reduced vertical transfer (and vice versa), confirming the implicit trade-off described in many models for the evolution of parasite virulence (damage to host fitness; MAY and ANDERSON 1983; BULL 1994; EWALD 1994). However, available host density did not mediate the trade-off because plasmids featuring increased (and reduced) conjugation evolved in all treatments (TURNER *et al.* 1998).

Failure of susceptible host density to mediate the trade-off in pB15 can be explained if plasmid spread does not increase in proportion to cell concentration. Here I examine the effects of cell density on horizontal transfer of pB15 and demonstrate that invasiveness decreases with cell concentration in static culture environments. In addition, I show the phenomenon is due to phenotypic plasticity in traits governing vertical and ho-

TABLE 1

Key bacterial strains used in this study

Strain	Relevant properties[a]	Abbreviation	Source
REL1206	Plasmid-free *Escherichia coli* B	Ara^-	Lenski *et al.* (1991)
REL1207	Spontaneous Ara^+ mutant of REL1206	Ara^+	Lenski *et al.* (1991)
REL5382	Transconjugant of REL1206 using pB15	Ara^-/pB15	Turner *et al.* (1998)
REL5384	Transconjugant of REL1207 using pB15	Ara^+/pB15	Turner *et al.* (1998)
PET366	Transconjugant of REL1206 using R1-drd19	Ara^-/R1-drd19	This study
PET318	Spontaneous Nal^r mutant of REL1207	Ara^+/Nal^r	Turner *et al.* (1998)

[a] Ara^+ indicates the ability to grow on L-arabinose. Nal^r indicates resistance to nalidixic acid; all strains are Nal^s unless otherwise indicated.

rizontal transfer of the plasmid. At low cell densities pB15 maximizes horizontal transmission at the expense of vertical transfer, but at high cell densities it features the reverse.

MATERIALS AND METHODS

Strains: Table 1 lists strains used in this study. Hosts (kindly provided by R. Lenski, Michigan State University, East Lansing, MI) were derived from a single clone of *E. coli* B (REL1206), which evolved previously for 2000 generations in a glucose-limited environment (Lenski *et al.* 1991); this strain cannot grow on L-arabinose and is denoted Ara^-. Ara^+ and Ara^- strains form white and red colonies, respectively, on tetrazolium-arabinose (TA) indicator plates (Levin *et al.* 1977). The Ara marker is selectively neutral under the shaking and static culture conditions used here (Travisano 1997; Turner *et al.* 1998).

Plasmids were obtained from the laboratory of B. Levin (Emory University, Atlanta, GA). R1 is a large (~100 kb) well-described plasmid of the IncFII group, featuring a copy number of four to five copies per cell (Nordstrom *et al.* 1980). R1 (and many other plasmids) normally repress conjugative pilus synthesis, but upon transfer to a new cell the plasmid's transfer operon is transitorily derepressed for a short time to facilitate further transmission (Willetts 1974; Lundquist and Levin 1986). In contrast, plasmid R1-drd19 used in this study is a mutant of R1 that is permanently derepressed for conjugation. R1-drd19 confers clinical resistance to kanamycin (Km) and several other antibiotics (Lundquist and Levin 1986). Plasmid pB15 is also large (~50 kb), but its copy number per cell is unknown. Although pB15 is not fully characterized, preliminary sequence data suggest it is related to R64, an IncI1 plasmid of Salmonella (D. Guttman and P. Turner, unpublished results). Plasmid pB15 conjugates at high rates in chemostats containing $\sim 10^8$ cells/ml in 50 μg/ml glucose medium (Lundquist and Levin 1986). It confers clinical resistance to Km and subclinical resistance to tetracycline (Tc; Lundquist and Levin 1986), confirmed by growth on TA with 25 μg/ml Km and 1 μg/ml Tc, respectively. To move a plasmid into REL1206, this recipient was mixed with plasmid-bearing donors, and transconjugants were obtained through overnight matings. No differences in plating efficiency on selective and nonselective media were observed for any of the plasmid-bearing cells in this study.

Plasmid segregation is incomplete transfer to one of the daughter cells during binary fission. R1-drd19 features at least one mechanism to ensure its stability in the absence of selection for plasmid carriage (Gerdes *et al.* 1986). It is unknown whether pB15 features a stability mechanism such as postsegregational killing (Helinski *et al.* 1996), but pB15 segregants are fairly rare; for strains REL5382 and REL5384 (Table 1), ~1 in 4000 colonies formed on TA is a segregant on the basis of tooth-picking assays onto TA + Km (Turner *et al.* 1998). Thus, both pB15 and R1-drd19 can be considered relatively stable under the current culture conditions.

Culture conditions: Bacteria were grown at 37° in batch culture using Davis minimal (DM) broth (Carlton and Brown 1981) supplemented with 2 μg/ml thiamine hydrochloride and glucose (glu) at a specific concentration; *e.g.*, DM25 indicates DM broth with 25 μg/ml glu, which yields $\sim 5 \times 10^7$ cells/ml at stationary phase. Culture volume was 10 ml, in nonshaking 18 × 150-mm glass tubes or shaking 50-ml Erlenmeyer flasks. Daily propagation occurred by vortexing a culture, followed by 100-fold dilution into fresh medium (serial transfer). During this 24-hr cycle, bacteria attained stationary-phase densities, at which point they had depleted the available resource. The resulting 100-fold growth of the population represents ~6.64 ($= \log_2 100$) generations of binary fission per day.

Invasion-when-rare experiments: To examine plasmid spread, donors and recipients were mixed ~1:200 in DM broth containing 10, 12.5, 25, 50, 100, 200, 400, 800, or 1000 μg/ml glu. Mixtures were serially transferred for up to 20 days, in the presence or absence of shaking. Every day, after serial transfer had taken place, glycerol was added to each population, which was then stored in a freezer at −80° for future study. Daily samples were plated on TA to track recipient densities and on TA with 25 μg/ml Km to measure densities of donors and transconjugants. (Transconjugants were also tested on TA with 1 μg/ml Tc and no dissociation between resistance markers was observed, indicating that plasmids did not lose the Km marker over time as they were transferred between cells.) In some experiments, populations were sampled on minimal-arabinose (MA) plates containing Km, to screen for Ara^+ transconjugants that were very rare relative to Ara^- donors. Km is an aminoglycoside that is bactericidal to sensitive cells, as confirmed by spreading plasmid-free recipients onto TA + Km. Therefore, transconjugant estimates were not confounded by matings between recipients and plasmid-bearing cells on TA + Km plates. To further dismiss plate matings as a potential confounding factor, diluted samples from frozen populations were spread on both TA + Km and MA + Km plates; colony counts yielded identical estimates of transconjugant densities per milliliter.

Conjugation rate assay: To assay conjugation rate (γ), Ara^-/pB15 donors and Ara^+ Nal^r (nalidixic acid resistant) recipients were mixed ~1:100 and allowed to grow and mate during a standard 24-hr growth cycle in static culture. The Nal^r marker facilitated visualization of rare transconjugants on selective plates containing 25 μg/ml Km and 15 μg/ml Nal. After 24 hr, the final densities of donors (D), recipients (R), and transconjugants (T) were determined by colonies formed on selective

and nonselective plates. Growth rate per hour in exponential phase (ψ) of mating cultures was estimated by regressing the natural logarithm of total cell density *vs.* time during the period of exponential-phase growth. The rate of conjugative transfer (milliliters per cell hour) for matings in batch culture may be estimated using the formula

$$\gamma = \psi \times \ln[1 + (T/R)(N/D)]/(N - N_0)$$

(Simonsen *et al.* 1990), where $N = T + R + D$ and N_0 is initial population size. Unlike other transfer measures (*e.g.*, Watanabe 1963; Bale *et al.* 1987), the end-point method is largely unaffected by factors such as donor-to-recipient ratio because it estimates the actual rate constant of transfer rather than simply the resulting frequency of transconjugants (Simonsen *et al.* 1990). The equation is simplified by using ψ for the total population, thereby ignoring predictably slower growth of plasmid-bearing cells. However, even reasonably large costs of carriage do not strongly affect estimation of γ (Simonsen *et al.* 1990).

Relative fitness and cost of plasmid carriage: To estimate relative fitness, two strains (distinguished by the Ara marker) were competed under the culture regimes described above. Strains were grown separately (preconditioned) for 1 day in the experimental medium to ensure comparable physiological states. They were then mixed 1:1, diluted 1:100 into fresh medium, and allowed to grow and compete for 24 hr. Initial and final densities of each competitor were estimated on TA plates.

Let the initial densities of the two competitors be $N_1(0)$ and $N_2(0)$, respectively, and let $N_1(1)$ and $N_2(1)$ be their densities after 1 day. The time-average rate of increase, m_i, for each competitor was then calculated as

$$m_i = \ln[N_i(1)/N_i(0)]/(24 \text{ hr}).$$

The fitness of one strain relative to the other is expressed simply as the dimensionless ratio of their rates of increase:

$$W_{ij} = m_i/m_j$$

(Lenski *et al.* 1991). Cost of plasmid carriage was measured by competing a plasmid-bearing strain against its plasmid-free counterpart that differed by the neutral Ara marker. Fitness of the plasmid-bearing strain relative to the plasmid-free strain was calculated as above. Cost of carriage (c) is the difference between 1.0 and the estimated fitness; thus, $c > 0$ indicates that a plasmid reduces host fitness. Fitness estimates are complicated, in principle, by segregants and transconjugants that arise during competitions between plasmid-bearing and plasmid-free cells. To ensure accurate estimates, I sampled a subset of cells to track plasmid losses and gains due to segregation and conjugation that occurred during competitions. As previously observed (Turner *et al.* 1998), segregants and transconjugants were small minorities, and their inclusion or exclusion from the calculations had no significant effect on fitness estimates.

RESULTS

Invasion by pB15 is maximal at intermediate densities in static culture: Invasion-when-rare experiments in static environments were used to examine the effects of cell density on spread of plasmid pB15. In these assays the minority donors (Ara^+/pB15) should decline due to the cost of plasmid carriage, whereas the majority recipients (Ara^-) should remain roughly constant (unless Ara^-/pB15 transconjugants become very numerous). These predictions were generally supported, confirming that pB15 was costly in all treatments (Figure 2). In some cases, donors decreased to low densities where (presumably) their loss due to selection was balanced by their increase through reinfection of rare segregants.

Invasion success was gauged by tracking transconjugant densities. Results (Figure 2) showed that the rate of transconjugant formation per day was not sufficient to outpace selection against plasmid carriage at 12.5 glu (linear regression with slope = −0.1408, t = 1.886, d.f. = 4, P = 0.132) and 25 glu (slope = −0.0789, t = 3.452, d.f. = 7, P = 0.011). (Assay at 12.5 glu was halted at 11 days due to contamination, but by this time transconjugants were already below the limit of detection on TA + Km.) Consistent with the predicted effect of increased cell density, pB15 invasion improved at intermediate glucose concentrations: 50 glu (slope = 0.0979, t = 13.629, d.f. = 14, $P < 0.0001$) and 100 glu (slope = 0.0944, t = 14.139, d.f. = 14, $P < 0.0001$). But at high concentrations, the plasmid invaded poorly: 200 glu (slope = −0.0173, t = 5.405, d.f. = 15, $P < 0.0001$), 400 glu (slope = −0.1287, t = 7.555, d.f. = 15, $P < 0.0001$), and 800 glu (slope = −0.0888, t = 3.523, d.f. = 14, P = 0.003). Transconjugants disappeared faster than donors in the latter two environments. This result could occur if the cost of carriage is higher in Ara^- cells, but this idea seems unlikely given marker neutrality under the current conditions (Travisano 1997; Turner *et al.* 1998). A plausible explanation is that the Ara^+ donors are more numerous than the Ara^- transconjugants, causing the Ara^+ background to provide a larger segregant pool for reinfection. There was also a sharp drop in donor and transconjugant densities on day 12 in 400 glu. This decline could easily result from a pipette error causing >100-fold dilution during serial passage; the plasmid-bearing cells were in the minority and sampling error could cause them to be underrepresented in the propagated cells.

Figure 3A shows the data for rates of transconjugant formation ($\log_{10}$ transconjugants/ml/day) *vs.* mean $\log_{10}$ R (stationary-phase recipient density). Clearly, the invasion rate of pB15 is positive (and maximal) only at the intermediate resource concentrations. My results demonstrate that spread of pB15 in static culture does not increase in direct proportion to cell density, as governed by glucose concentration.

Invasion by pB15 is unaffected by chromosomal markers: The above results could be due to an unexpected interaction between pB15 and the Ara^- marker on the recipient chromosome. To examine this possibility, two experiments were conducted. First, Ara^-/pB15 donors invaded Ara^+ recipients for 9 days (sufficient time to verify previous dynamics) at three glucose concentrations. In these assays recipient densities remained approximately constant, whereas donors declined due to plasmid carriage (data not shown). More importantly, the rate of transconjugant formation per day (Figure 3A) was qualitatively similar to that of the above assays involving the opposite

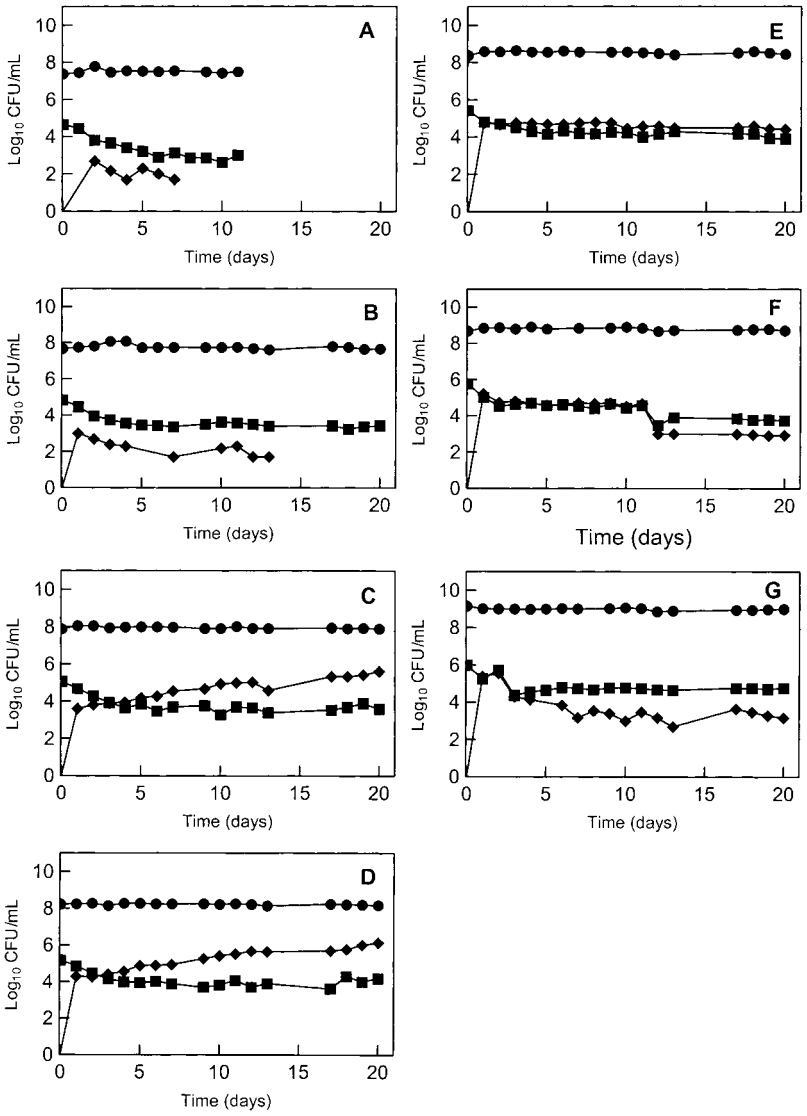

FIGURE 2.—Invasion by plasmid pB15 occurs only at intermediate host densities in static culture. Log_{10} colony-forming units (CFU) per milliliter of Ara^- recipients (circles), Ara^+/pB15 donors (squares), and Ara^-/pB15 transconjugants (diamonds) during serial transfer in seven environments are shown: (A) 12.5, (B) 25, (C) 50, (D) 100, (E) 200, (F) 400, and (G) 800 μg/ml glucose.

marker combination for donors and recipients: 25 glu (slope = −0.1258, d.f. = 7, $P = 0.009$), 100 glu (slope = 0.1461, d.f. = 7, $P = 0.001$), and 800 glu (slope = −0.1301, d.f. = 7, $P = 0.002$).

Second, invasion experiments were repeated at seven glucose concentrations for 10 days, but donors and recipients featured the identical Ara marker. These assays are less accurate because donors cannot be distinguished from transconjugants, but they can determine whether pB15 transfer between differently marked cells accounts for poor invasion at high cell densities. Consistency between these and the above assays would be maximal plasmid spread at intermediate glucose concentrations. However, the maximum rate can be net negative because majority donors can decline as minority transconjugants increase. Once again, results showed that the rate of plasmid spread was most rapid at intermediate glucose concentrations, regardless of the Ara marker shared by donors and recipients (Figure 3B).

Invasion by R1-drd19 is maximal at high densities in static culture: Failure of pB15 to invade fastest at high cell densities could be due to particulars of the culture regime. To examine this potential bias, I conducted invasion experiments using a different plasmid. Ara^-/R1-drd19 donors invaded Ara^+ recipients for 5 days at three glucose concentrations, with threefold replication. Results (Figure 4) showed that the recipients remained approximately constant, whereas the donors declined due to plasmid carriage. As cell density increased, the mean rate of change in transconjugants also increased: 10 glu (slope = −0.9283, d.f. = 1, $P = 0.181$), 100 glu (slope = 0.1742, d.f. = 3, $P = 0.001$), and 1000 glu (slope = 0.6299, d.f. = 3, $P = 0.0018$). These data showed that the static culture regime did not bias against increased plasmid spread at higher glucose concentrations.

Mass action governs invasion by R1-drd19 and pB15 in shaking environments: To examine whether mass action governs plasmid spread in shaking environments, I con-

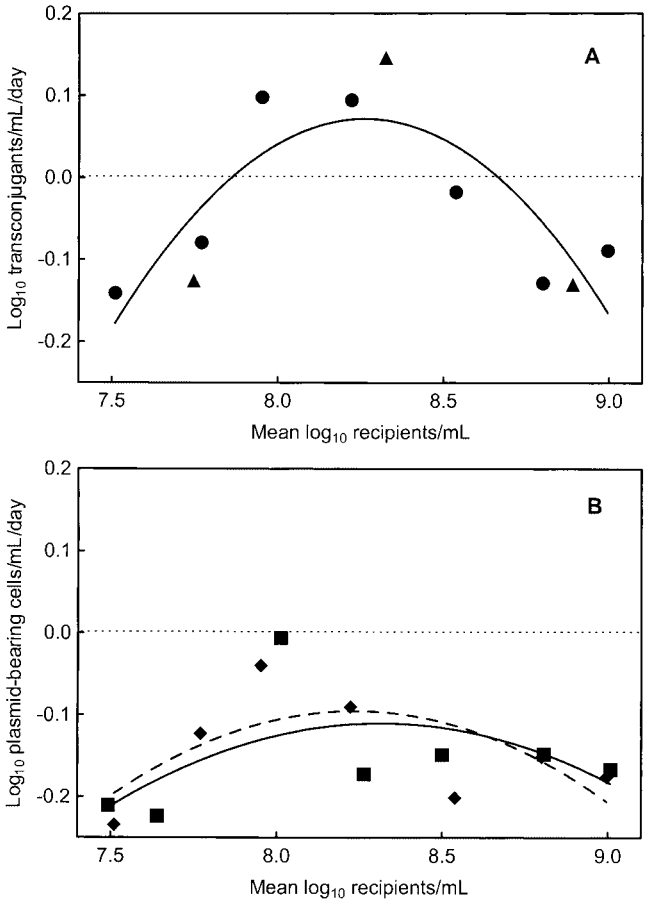

FIGURE 3.—Invasion by plasmid pB15 is maximal at intermediate host densities in static culture. (A) Solid circles summarize the experiments shown in Figure 2. Solid triangles are from a second set of invasion assays in fewer environments (25, 100, and 800 μg/ml glucose), using Ara^+ recipients and Ara^-/pB15 donors. The solid line is a quadratic fit to the data. (B) Seven independent invasion assays (12.5, 25, 50, 100, 200, 400, and 800 μg/ml glu), using Ara^--marked donor and recipient cells (solid squares, dashed line) and using Ara^+-marked donors and recipients (solid diamonds, solid line). See text for details.

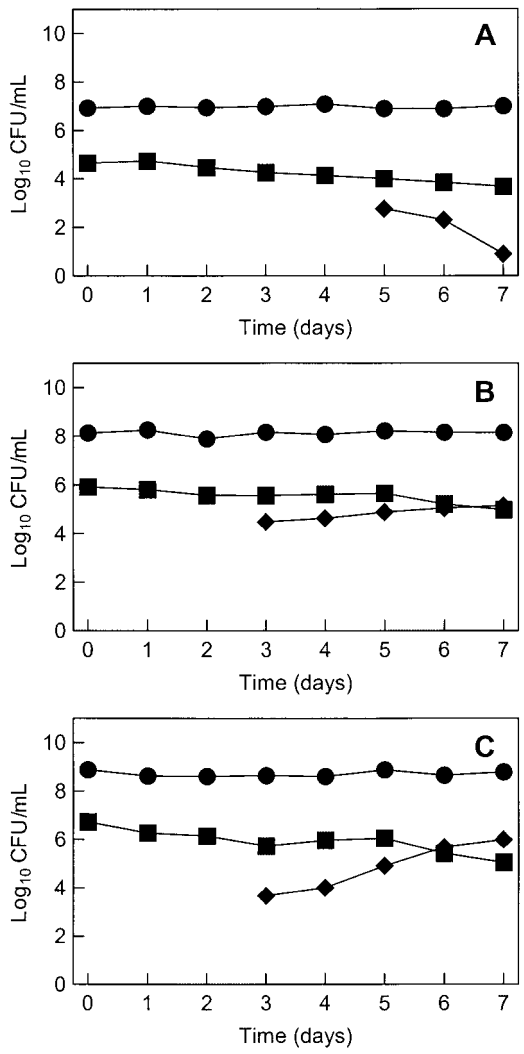

FIGURE 4.—Invasion by plasmid R1-drd19 occurs in proportion to host density in static culture. Each point represents the mean of independent assays ($n = 3$). Log_{10} CFU/ml of Ara^+ recipients (circles), Ara^-/R1-drd19 donors (squares), and Ara^+/R1-drd19 transconjugants (diamonds) during serial transfer in three environments are shown: (A) 10, (B) 100, and (C) 1000 μg/ml glucose.

ducted invasions using R1-drd19 and pB15 in shaking culture. Ara^-/R1-drd19 donors invaded Ara^+ recipients, whereas Ara^+/pB15 donors invaded Ara^- recipients. Both mating combinations were replicated threefold, at 100 and 1000 glu. Results (Figure 5) showed that for R1-drd19 the mean rate of change in transconjugants per day was faster at high glucose concentration, similar to the outcome in static culture: 100 glu (slope = 0.5578, d.f. = 3, $P = 0.024$) and 1000 glu (slope = 0.6179, d.f. = 3, $P = 0.017$). More importantly, the data revealed that pB15 can successfully invade at intermediate *and* high densities in shaking environments: 100 glu (slope = 0.7921, d.f. = 2, $P = 0.031$) and 1000 glu (slope = 0.8264, d.f. = 3, $P = 0.0048$). These results indicated that mass action is a reasonable descriptor of pB15 spread in well-agitated liquid culture.

Conjugation rate of pB15 declines at high densities in static environments: The horizontal component of plasmid spread (γR, see Introduction) is assumed to be proportional to the density of potential recipients (R). Because this prediction for pB15 breaks down in static culture (Figure 3), it suggests that conjugation rate (γ) or host density (R) (or both) deviates from expectations.

I first tested whether glucose concentration predictably governs R in static culture. To do so, I calculated mean $\log_{10} R$ at each glucose concentration using the data from pB15 invasions in static environments (Figure 2): 12.5 glu, 3.24×10^7 cells/ml; 25 glu, 5.88×10^7 cells/ml; 50 glu, 8.95×10^7 cells/ml; 100 glu, 1.67×10^8 cells/ml; 200 glu, 3.45×10^8 cells/ml; 400 glu, 6.30×10^8 cells/ml; and 800 glu, 9.90×10^8 cells/ml. This analysis showed that cell density approximately doubled

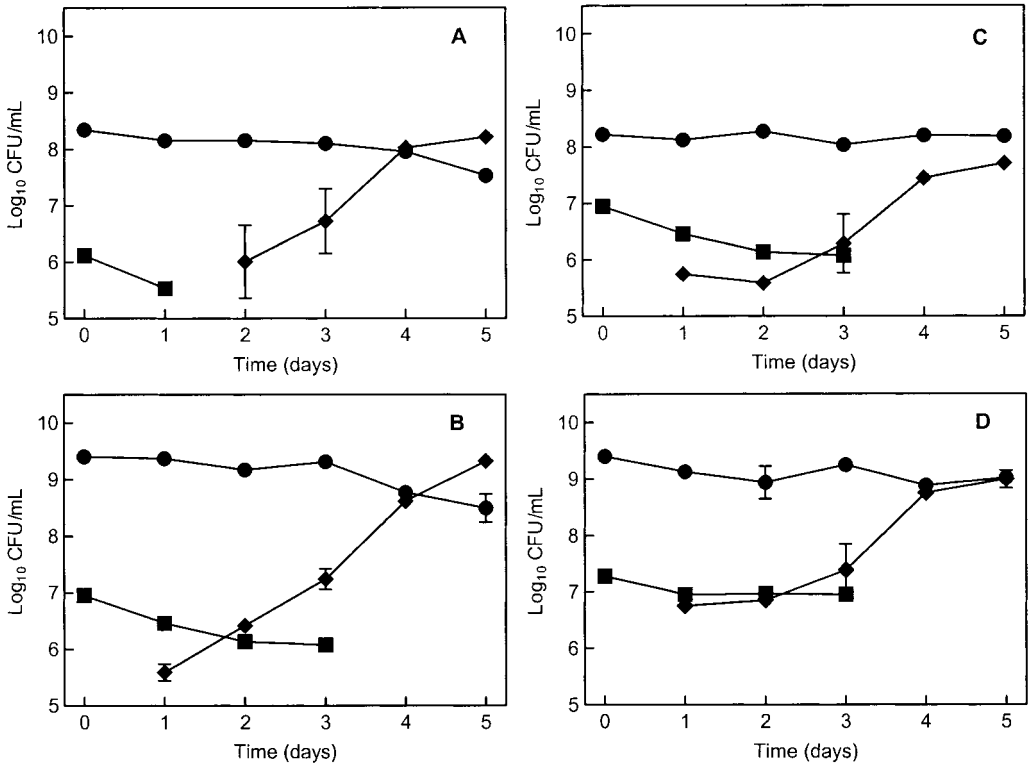

FIGURE 5.—Invasions by plasmids R1-drd19 and pB15 occur in proportion to host density in shaking culture. Each point represents the mean (±SE) of independent assays ($n = 3$); error bars too small to be visualized are omitted. (A and B) Log_{10} CFU/ml of Ara^+ recipients (circles), Ara^-/R1-drd19 donors (squares), and Ara^+/R1-drd19 transconjugants (diamonds) during serial transfer in two environments: (A) 100 and (B) 1000 μg/ml glucose. (C and D) Identical experiments using Ara^- recipients (circles), Ara^+/pB15 donors (squares), and Ara^-/pB15 transconjugants (diamonds): (C) 100 and (D) 1000 μg/ml glucose.

as the glucose concentration similarly increased, and a linear regression of mean $\log_{10} R$ on glucose concentration was statistically significant (slope is 0.0017, d.f. = 5, $t = 4.046$, $P = 0.0099$).

To examine whether pB15 conjugation is constant across cell densities, I measured γ at 5, 50, 500, and 1000 glu in static culture; six blocks of assays were performed. Results showed that γ was highly sensitive to cell density governed by glucose concentration (Figure 6A). A two-way ANOVA showed a highly significant effect of glu on γ, but no block effect (Table 2). Mean γ ($n = 6$) for pB15 at 5 glu was 1.092×10^{-10} ml/cell hour; that is, during 1 hr, each plasmid-bearing cell can effectively "search" a volume of $\sim 10^{-10}$ ml and infect any plasmid-free cell therein. (Because cell densities are $\ll 1/\gamma$, a donor is unlikely to encounter two recipients in close temporal proximity, or vice versa; hence, the system is unsaturated.) In contrast, mean γ progressively diminished at 50 glu (5.315×10^{-11} ml/cell hour), 500 glu (6.185×10^{-12} ml/cell hour), and 1000 glu (9.483×10^{-13} ml/cell hour). To further examine this phenomenon, I estimated mean R ($n = 6$) at each glucose concentration: 5 glu, 1.65×10^7 cells/ml; 50 glu, 8.81×10^7 cells/ml; 500 glu, 8.76×10^8 cells/ml; and 1000 glu, 2.16×10^9 cells/ml. Using mean values of γ and R, it is revealed that γR in pB15 does not increase with glucose concentration. Rather, γR is approximately constant between low and intermediate cell densities (5 glu, 0.00542; 50 glu, 0.00533) and even declines slightly at the highest experimental densities (500 glu, 0.00180; 1000 glu, 0.00205). This phenomenon occurs because γ is not constant, but declines by approximately an order of magnitude as R increases by the same amount.

Trade-off between vertical and horizontal transfer of pB15 holds across cell densities: Because vertical and horizontal transfer routes in pB15 show a trade-off (TURNER *et al.* 1998), the cost of plasmid carriage should vary along with γ across glucose concentrations. To test this hypothesis, I measured the fitness of Ara^+/pB15 cells relative to Ara^- cells in seven glucose concentrations (12.5, 25, 50, 100, 200, 400, 800, and 1000 glu) in static culture, with replication ($n = 2$). I then calculated the cost of plasmid carriage, c (see MATERIALS AND METHODS). Results (Figure 6B) indicated that plasmid-bearing cells were generally disadvantaged, as all values of c exceeded zero. More importantly, these data showed that c decreased at higher glucose concentrations, and this outcome was statistically significant (linear regression with slope = −0.1341, d.f. = 14, $t = 4.487$, $P = 0.0005$). These data demonstrate that the genetic trade-off between transmission modes in pB15 holds across a wide range of cell densities and that both γ and c are phenotypically plastic in static culture containing glucose.

DISCUSSION

Many parasites can transfer vertically between parent and offspring, as well as horizontally between infected and uninfected individuals. Activities that augment horizontal transmission (such as greater within-host reproduction) are assumed to reduce host fitness, thereby decreasing vertical transmission (MAY and ANDERSON

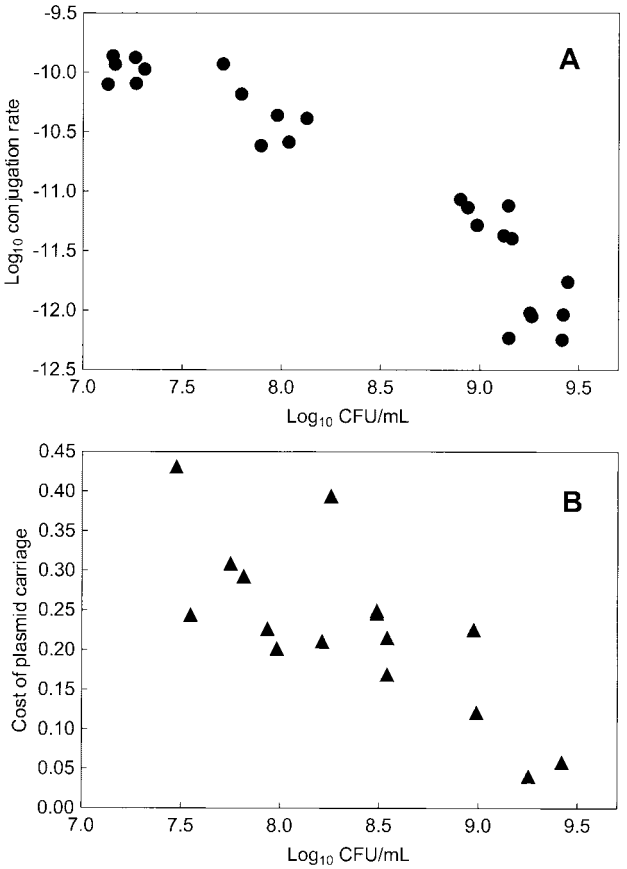

FIGURE 6.—Plasmid pB15 features phenotypically plastic traits in static culture environments. Each point represents an independent measurement. (A) Conjugation rate (solid circles) of pB15 decreases with increasing cell concentration (Log_{10} CFU/ml). See Table 2 for statistics. (B) Cost of plasmid carriage (solid triangles) similarly declines, due to the trade-off between horizontal and vertical modes of transmission. See text for statistical analyses.

1983; HERRE 1993; BULL 1994; EWALD 1994; but see EBERT and BULL 2003). We previously observed that plasmid pB15 evolves greater conjugation at the expense of reduced intergenerational transfer (and vice versa) in static culture (TURNER *et al.* 1998), demonstrating the trade-off between transmission modes commonly assumed in theories for the evolution of parasite virulence (see also DIFFLEY *et al.* 1987; DEARSLEY *et al.* 1990; BULL *et al.* 1991; EBERT and MANGIN 1997; MESSENGER *et al.* 1999). Although the models predict that susceptible host abundance should determine which transfer mode is selectively favored, host density failed to mediate the trade-off in pB15 because plasmids evolved increased (and reduced) conjugation in all treatments (TURNER *et al.* 1998).

TABLE 2

ANOVA of the effect of glucose concentration on conjugation rate (γ) of plasmid pB15

Source	SS	d.f.	MS	*F*	*P*
Glucose concentration	15.673	3	5.224	193.011	<0.0001
Block	0.233	5	0.047	1.724	0.1897
Error	0.406	15	0.027		

SS, sum of squares; MS, mean square.

Theory generally predicts that plasmid spread should improve with increasing cell density due to greater contact between donor and recipient cells (*e.g.*, STEWART and LEVIN 1977; LEVIN *et al.* 1979; SIMONSEN 1990; LAGIDO *et al.* 2003). Here I demonstrated that host density failed to mediate the trade-off in pB15 because the plasmid's rate of horizontal spread is not proportional to cell density in static culture. Rather, invasion by pB15 is maximal at intermediate cell densities determined by glucose concentration. This result explains why manipulation of available host density at elevated glucose concentrations (*i.e.*, $\sim 10^9$ cells/ml, 1000 μg/ml glu) did not predictably select for the evolution of conjugation rates in pB15-derived plasmids (TURNER *et al.* 1998; see further discussion below).

Spread of pB15 occurs disproportionately to cell density in static culture because traits governing its transfer are phenotypically plastic. The rate of conjugative transfer (γ) is expected to be a constant, which is relatively insensitive to cell density, donor-to-recipient ratio, and other environmental factors (SIMONSEN *et al.* 1990). Thus, horizontal spread should be determined by the product of γ and susceptible host density (*R*, a variable determined by resource concentration), causing γ*R* to increase in direct proportion to *R*. In contrast, γ*R* in pB15 is relatively unchanged as *R* increases, because γ declines by approximately an order of magnitude as *R* is similarly increased in static culture containing glucose. Although it is clear that increased cell densities negatively impact spread of pB15 in static culture, it is questionable whether the mass-action measurement γ should be used to describe the phenomenon. SIMONSEN (1990) showed that measurements of γ are strikingly similar for plasmid R1-drd19 when bacteria are mated on surfaces and in shaking liquid culture, even when cells are grown at densities below those used in my experiments (*i.e.*, where donors very rarely encounter recipients). Therefore, the data by SIMONSEN (1990) suggest that γ may be usefully applied in static culture environments where matings occur both in liquid and between cells that settle out in culture. However, non-mass-action models should more accurately describe conjugal transfer in complex environments such as on surfaces (*e.g.*, LAGIDO *et al.* 2003).

At intermediate densities horizontal transfer in pB15 is sufficient to overcome the growth disadvantage suffered by plasmid-bearing cells, but at high densities its poor conjugation prevents the plasmid from invading. My data allow a crude estimate of the cell densities permitting successful invasion. For pB15, the rate of change in transconjugants per milliliter per day is appar-

ently a nonlinear function of $\log_{10} R$ (Figure 3A), and a quadratic function provides a statistically significant fit to the data ($F_{[2,7]} = 7.0428$, $P = 0.0211$). This function is described by $dT/dt = -29.957 + 7.268 \times R - 0.439 \times R^2$, which can be solved for the two values of R where the curvilinear fit crosses a value of zero. This solution yields $R = 7.244 \times 10^7$ cells/ml and 4.571×10^8 cells/ml as the lower and upper boundaries, respectively, for successful invasion by pB15 in static culture. Although the estimate indicates a relatively narrow range of existence conditions for pB15, more thorough examination of invasion conditions can be explored using the parameters described in this study.

The cost of plasmid carriage in pB15 is also phenotypically plastic in static culture, because the fitness disadvantage suffered by plasmid-bearing cells declines as hosts become more numerous. Thus, the previously described trade-off between transmission modes (Turner *et al.* 1998) is now revealed to hold across a broad range of cell concentrations in static culture. However, the plasticity observed in pB15 seems nonintuitive because it indicates that the plasmid relies more heavily upon vertical transfer as cell densities are increased.

Adaptive *vs.* nonadaptive phenotypic plasticity: Phenotypic plasticity may or may not be the result of adaptation (see Scheiner 1993 for review). If plasmid transfer is proportional to cell density, it seems logical that "optimal" phenotypic plasticity should allow increased plasmid virulence (more rapid conjugation) at high host densities and decreased virulence (reduced conjugation) at low host densities. Precisely the opposite was observed in pB15, suggesting the plasticity is nonadaptive. However, the theorized importance of cell density for plasmid spread is a reasonably good predictor for invasiveness of plasmid R1-drd19 in static and shaking environments and of pB15 in shaking culture. These combined results strongly suggest that an interaction between pB15 and the static culture environment governs the plasticity observed.

One possibility is that the plasticity is a coincidence of the novel association between plasmid pB15 and *E. coli* B bacteria. The experimental host was evolved *in vitro* for 2000 generations (Lenski *et al.* 1991), whereas pB15 was isolated from an unknown *E. coli* host taken from a human undergoing antibiotic treatment (Lundquist and Levin 1986). It may be that an epistatic interaction between plasmid and chromosomal genes is responsible in part (or wholly) for the observed plasticity. To examine this hypothesis, one could look for phenotypic plasticity when the plasmid is moved to a different host background, such as *E. coli* K-12 or unevolved *E. coli* B. Equally intriguing would be to examine the "cost of plasticity" in pB15, by predicting that evolution of plasmid/host associations in a constant environment would select against maintenance of phenotypic plasticity, assuming there is a cost of maintaining the associated genetic machinery; Drosophila experiments demonstrate that plasticity of a trait can change in response to selection (see Scheiner 1993 for review). This idea could be studied by evolving *E. coli* B/pB15 associations for prolonged periods in static culture at a single glucose concentration. In fact, this experiment has already been completed in the control populations from Turner *et al.* (1998), where replicated bacteria/plasmid associations were allowed to evolve for 500 generations in 1000 μg/ml glu. Thus, evolved plasmids from the prior study could be examined for changes in plasticity.

A second possibility is that the phenotypic plasticity is a consequence of differing physiology of the *E. coli* host at varying glucose (and hence host density) levels. The 2000 generations of evolution experienced by the bacterium at 25 μg/ml glu in shaking culture (Lenski *et al.* 1991) are sufficient for profound adaptive changes, which can cause very different performance in other environments (*e.g.*, Travisano 1997). Therefore, it is possible that the host's physiology could be greatly affected by growth in elevated glucose concentrations and/or static culture. In turn, because plasmids are obligate intracellular elements, expression of plasmid genes (such as conjugative ability) can be strongly affected by the host's physiological state (Levin *et al.* 1979). This idea is supported by the phenomenon of transitory derepression. Immediately after conjugation has occurred, the mechanism allows a plasmid to temporarily derepress its conjugation to maximize the rate of further horizontal transfer, suggesting transitory derepression occurs in response to host physiology.

At this juncture, it appears that pB15's conjugation rate is strongly influenced by levels of cell density in static culture. Preliminary sequence data for pB15 (D. Guttman and P. Turner, unpublished data) suggest that the plasmid may be related to R64, a large (~120 kb) IncI1 plasmid of *Salmonella typhimurium* (Hedges and Datta 1973). IncI1 plasmids form two types of sex pili, a thin flexible pilus and a thick rigid pilus (Bradley 1983, 1984). The thick pilus is essential for mating in general, whereas the thin pilus is required only for liquid mating. R64 features an elaborate 54-kb transfer mechanism, the R64 shufflon, which is prone to stochastic DNA rearrangements that strongly influence mating efficiency, especially recipient specificity in liquid culture (Komano *et al.* 1995; Komano 1999). Although pB15 appears genetically similar to R64, its transfer mechanism is probably much less complex because the entire pB15 genome (~50 kb) is smaller than the R64 shufflon (D. Guttman and P. Turner, unpublished data). However, pB15 might also feature two (or more) types of sex pili, and the influence of cell density and/or glucose concentration on the expression of genes related to pilus formation in pB15 is unknown. For instance, these factors might cause pB15 to differentially express one or more of its pilus genes, thereby influencing invasiveness. Because this mechanistic explanation is highly speculative,

I discuss several other possibilities below. In addition, I relate my data to previous studies in plasmid biology.

Potential mechanisms for density-dependent conjugation: A simple explanation for the density-dependent effect is that the conjugation process in pB15 somehow becomes "saturated" at higher cell densities in static culture, much as bacterial growth reaches a maximum rate that cannot be raised by increasing the concentration of a limiting resource (Monod 1949). However, saturation kinetics cannot explain the apparent maximum rate of plasmid increase at intermediate densities, as observed here. A similar possibility is that conjugation rate in pB15 is not a function of cell density *per se* but instead responds to the concentration of glucose, which was varied to manipulate cell density. That is, higher concentrations of glucose may somehow inhibit the conjugal transfer of pB15.

I examined this possibility by looking closely at the population dynamics occurring in the 24-hr mating experiments used to estimate conjugation rate. There was some evidence that the number of transconjugants increases unexpectedly during the transition from exponential growth to stationary phase, irrespective of cell density, suggesting that conjugation in pB15 is somehow stimulated by the depletion of glucose (data not shown). In particular, the number of transconjugants measurably increased between 8 and 10 hr of the growth cycle, whereas by this time the donors and recipients had evidently made the transition from exponential growth to stationary phase as the medium was being depleted of glucose. Simple models assume that the rates of bacterial growth and plasmid transfer are Monod functions (Monod 1949) of resource concentration, so that when resources are exhausted growth and conjugation cease (Stewart and Levin 1977; Simonsen *et al.* 1990). Thus, the conjugation rate of a plasmid is expected to be proportional to the growth rate of the mating population. However, in IncP plasmids the expression of transfer proteins can be highest when host cells are growing slowly and thus when vertical transfer of plasmids is minimized (Pansegrau *et al.* 1994). Further experiments could examine the dynamics of 24-hr matings with pB15 and the possibility of unexpectedly high transfer of the plasmid during the transition to stationary phase in static culture.

High cell densities (or high glucose concentrations) might strongly impact other plasmid or bacterial traits in my experiments. For instance, the average number of pB15 copies per cell might somehow decline at high densities in static environments, due to changes in the host (*e.g.*, replication control during cell division) or in plasmid regulation of copy number. But this explanation works only if changes in plasmid copy number affect infectiousness (*e.g.*, if increased copy number increases conjugative pilus formation), and to my knowledge this link has not been documented. Similarly, the level of carbon source might negatively impact the motility of cells in my static-culture experiments, thus reducing the potential for transfer to occur at high cell densities. This idea could explain the pB15 results, but seems unlikely given that transfer of R1-drd19 was not similarly hampered by elevated densities in static culture.

Relevance to previous work: Most models of plasmid transfer depend on simple mass-action kinetics, but it is now widely recognized that the majority of bacteria found in natural, clinical, and industrial settings persist in association with surfaces (Davey and O'Toole 2000). Therefore, plasmid conjugation is likely to be a complex process that extends beyond simple models (*e.g.*, Lagido *et al.* 2003). Further complexities may arise because conjugation can be sensitive to both abiotic and biotic conditions (Fernandez-Astorga *et al.* 1992), such as temperature (Fernandez-Tresguerres *et al.* 1995) and dissimilarities between donor and recipient strains (*e.g.*, Dionisio *et al.* 2002). Pappas and Winans (2003) showed that conjugation of the Ti plasmid is activated by quorum sensing in the phyto-pathogen *Agro-bacterium tumefaciens*, demonstrating density-dependent changes in plasmid transfer ability. Thus, earlier findings indicate that phenotypic plasticity can occur in plasmids, but to my knowledge the data were never described in light of this important biological phenomenon.

Virulence models predict that virulent parasites can evolve at increased host densities, because available host abundance favors selection for highly infectious genotypes. The underlying assumption is that increased virulence leads to reduced host fitness, thereby preventing a parasite from simultaneously maximizing vertical *and* horizontal transfer. We chose plasmid pB15 to examine this hypothesis (Turner *et al.* 1998), on the basis of its ability to successfully invade *E. coli* K-12 populations growing at $\sim 10^8$ cells/ml in chemostat culture (Lundquist and Levin 1986). To do so, we used static serial-culture environments containing 1000 μg/ml glu ($\sim 10^9$ cells/ml). Our previous experiment was correctly designed to test the theoretical prediction, in principle. Preliminary experiments with pB15 showed that the plasmid can invade when rare at moderate cell densities ($\sim 10^8$ cells/ml; 50 μg/ml glu) in static culture (Turner 1995), as confirmed here. However, my current data also reveal that the combination of plasmid pB15 and a glucose-rich static culture regime was an unfortunate choice, because this environment negatively impacts the plasmid's conjugation ability (*i.e.*, R1-drd19 would have been an okay choice because its spread improves with increasing cell density in the presence and absence of shaking). Hence, we may have created weak ability for host density to select for conjugation in our prior study, resulting in the apparently stochastic evolution of increased (and reduced) conjugation in all of our treatments. We can be criticized for conducting our study at 1000 μg/ml glu, without first examining the associated invasion dynamics of pB15 (Turner *et al.* 1998); otherwise we might have eliminated pB15 or static culture as

suitable choices. But the negative effect of cell density on conjugation rate of pB15 in static culture is both unusual and unexpected. Therefore, we had no *a priori* reason to believe that a 20-fold increase in cell density would inhibit the invasiveness of pB15 seen in our preliminary assays (Turner 1995).

Whatever the precise mechanistic explanation for the observed phenomena, they may explain the failure of a simple model to predict the evolutionary response of pB15 to experimental manipulations of susceptible host density. Although the *genetic* assumption of a trade-off between rates of horizontal and vertical transmission was fulfilled (Turner *et al.* 1998), the *ecological* assumption that the rate of horizontal transmission is simply proportional to susceptible host density was evidently not satisfied (current findings). More generally, models of phenotypic evolution depend on both genetic and ecological assumptions, and the predictions of these models may fail as a consequence of violating either type of assumption.

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