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Stability of a promiscuous plasmid in different hosts: no guarantee for a long-term relationship

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Broad-host-range (BHR) IncP-1 plasmids have the ability to transfer between and replicate in nearly all species of the *Alpha*-, *Beta*- and *Gammaproteobacteria*, but surprisingly few data are available on the stability of these plasmids in strains within their host range. Moreover, even though molecular interactions between the bacterial host and its plasmid(s) exist, no systematic study to date has compared the stability of the same plasmid among different hosts. The goal of this study was to examine whether the stability characteristics of an IncP-1 plasmid can be variable between strains within the host range of the plasmid. Therefore, 19 strains within the *Alpha*-, *Beta*- or *Gammaproteobacteria* carrying the IncP-1β plasmid pB10 were serially propagated in non-selective medium and the fraction of segregants was monitored through replica-picking. Remarkably, a large variation in the stability of pB10 in different strains was found, even between strains within the same genus or species. Ten strains showed no detectable plasmid loss over about 200 generations, and in two strains plasmid-free clones were only sporadically observed. In contrast, three strains, *Pseudomonas koreensis* R28, *Pseudomonas putida* H2 and *Stenotrophomonas maltophilia* P21, exhibited rapid plasmid loss within 80 generations. Parameter estimation after mathematical modelling of these stability data suggested high frequencies of segregation (about 0.04 per generation) or high plasmid cost (i.e. a relative fitness decrease in plasmid-bearing cells of about 15 and 40 %), which was confirmed experimentally. The models also suggested that plasmid reuptake by conjugation only played a significant role in plasmid stability in one of the three strains. Four of the 19 strains lost the plasmid very slowly over about 600 generations. The erratic decrease of the plasmid-containing fraction and simulation of the data with a new mathematical model suggested that plasmid cost was variable over time due to compensatory mutations. The findings of this study demonstrate that the ability of a so-called 'BHR' plasmid to persist in a bacterial population is influenced by strain-specific traits, and therefore observations made for one strain should not be generalized for the entire species or genus.

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INTRODUCTION

Comparative analyses of fully sequenced bacterial genomes suggest that horizontal gene transfer has played a significant role in the adaptive evolution of microbial life (Gogarten & Townsend, 2005; Gogarten *et al.*, 2002; Jain *et al.*, 2002). In particular, horizontal transfer (HT) of broad-host-range (BHR) antibiotic resistance plasmids through conjugation is important to the spread of drug resistance genes (de la Cruz & Davies, 2000; Frost *et al.*, 2005; Mazel & Davies, 1999), as these plasmids can transfer between and replicate in a broad range of taxonomically diverse species. Besides conjugation and replication, stability in the absence of selection for plasmid-encoded traits is a third characteristic that should be considered in the assessment of the long-term host range of a plasmid. Indeed, once a plasmid has transferred to and replicates in a new host, different long-term outcomes are possible. In the presence of selective pressure for one or multiple plasmid-encoded genes, the initial transconjugant can give rise to a plasmid-carrying population. In contrast, in the absence or after disappearance of selective pressure the plasmid may only transiently be retained if it is unstable in this new host (Smets & Barkay, 2005). In spite of the important impact of plasmid stability on the long-term host range of a plasmid, documenting plasmid persistence in various populations in the absence of selection has received surprisingly little attention.

Different processes lie at the basis of plasmid retention or loss in a bacterial population. In spite of the presence of active partitioning, multimer resolution and post-segregational

Abbreviations: BHR, broad-host-range; HGT, horizontal gene transfer; HT, horizontal transfer; SS, segregation selection; VS, variable selection.

killing systems encoded on some plasmids, segregants are still formed at very low frequencies (Helinski *et al.*, 1996; Nordström & Austin, 1989), and segregational loss can be higher if these systems do not properly function in certain hosts (Summers, 1991). Plasmids also often reduce the fitness of the host in the absence of selective pressure (for example, Dahlberg & Chao, 2003; Turner *et al.*, 1998, and references therein) and thus can impose a cost or 'burden' to the host. Therefore, even under very low frequencies of segregational loss, the fraction of plasmid-free segregants can increase rapidly through their differential growth advantage. On the other hand, conjugational plasmid transfer into these segregants shortly after they arise ('reinfection') may counter plasmid loss, and thus prevent a selective sweep of plasmid-free hosts. The combination of plasmid loss, conjugative transfer, plasmid cost and the presence of selection will therefore determine whether or not a plasmid can persist in a population over evolutionary time (Stewart & Levin, 1977).

Plasmids belonging to the IncP-1 group are thought to be among the most promiscuous plasmids known so far. They can transfer between and ensure vegetative replication in a wide variety of phylogenetically distinct genera, belonging to the *Alpha-*, *Beta-* and *Gammaproteobacteria* (Guiney & Lanka, 1989; Krishnapillai, 1988; Thomas & Smith, 1987; Thomas & Helinski, 1989). Although the general statement that 'IncP-1 plasmids are capable of stable maintenance in almost all Gram-negative bacteria', first stated by Thomas & Smith (1987) and Thomas & Helinski (1989), is readily copied from these reviews or others (Adamczyk & Jagura-Burdzy, 2003), we found that some of the most cited studies to support this claim (Datta & Hedges, 1972; Datta *et al.*, 1971; Olsen & Shipley, 1973) did not present data that unequivocally demonstrated plasmid stability in various hosts. It is known, however, that most plasmids rely extensively on the host replication machinery (Espinosa *et al.*, 2000; Toukdarian, 2004), and interactions between the host and plasmid have important implications for the ability of plasmids to colonize new hosts (del Solar *et al.*, 1996). A role for host factors in other plasmid-related processes, such as conjugative transfer and plasmid partitioning, has been suggested but actual information is quite limited (Koraimann, 2004; Williams & Thomas, 1992). Thus although the host cell is the main environment for the plasmid, the long-term persistence of promiscuous plasmids in taxonomically different hosts in the absence of selective pressure is unknown (Diaz Ricci & Hernández, 2000).

To improve our understanding of plasmid stability in strains within the host range of a BHR plasmid, we examined the stability of the multiresistance IncP-1β plasmid pB10 (Dröge *et al.*, 2000; Schlüter *et al.*, 2003) in 19 different strains of *Alpha-*, *Beta-* or *Gammaproteobacteria*. From these data, the segregational frequency, plasmid cost and HT frequency were estimated through mathematical modelling and statistical analysis (Ponciano *et al.*, 2007). Our results showed a wide variety of plasmid population dynamics in different hosts, ranging from 100 % plasmid retention to rapid plasmid loss. Moreover, plasmid stability patterns were not correlated with phylogenetic relatedness of the hosts, but seemed to be strain-specific.

METHODS

Bacterial strains and plasmid. The 64.5 kb plasmid pB10 (Schlüter *et al.*, 2003), isolated from the bacterial community of a wastewater treatment plant (Dröge *et al.*, 2000), is a self-transmissible, BHR IncP-1β plasmid that mediates resistance against the antibiotics tetracycline (Tc), streptomycin (Sm), amoxycillin and sulfonamide, and against mercury ions.

The 19 strains used in the study are listed in Table 1. For all strains used, the concentrations of Tc and Sm that were necessary to prevent growth of plasmid-free cells, but allowed growth of plasmid-containing cells were determined by streaking a small aliquot from a 24 h Luria–Bertani (LB) broth (Sambrook & Russell, 2001) culture onto LB agar plates (15 g agar l^{-1}) with different concentrations of the antibiotics. The concentrations of Tc and Sm selecting for plasmid carriage in each strain were: 25 μg Tc ml^{-1} and 1000 μg Sm ml^{-1} for EEZ23, C17, S96, S100, S55, P18, AE815 and RM1021; 25 μg Tc ml^{-1} and 250 μg Sm ml^{-1} for R28, UWC1, H2, S60, S37 and C20; 25 μg Tc ml^{-1} and 50 μg Sm ml^{-1} for K12 and R16; 100 μg Tc ml^{-1} and 250 μg Sm ml^{-1} for PAO1, R39 and S34; and 100 μg Tc ml^{-1} and 1000 μg Sm ml^{-1} for P21. The strains obtained during our previous study (De Gelder *et al.*, 2005) are activated sludge bacteria that acquired an *rfp*-marked variant of pB10, except for C17 and C20, which were sludge bacteria isolated on R2A agar (Table 1). From the sludge isolates with plasmid pB10::rfp, non-fluorescent plasmid-free colonies were obtained through growth in plain LB, followed by plating and purification on LB agar. Subsequently, spontaneous mutants resistant to rifampicin (Rf) were obtained by transferring an aliquot of an LB culture into LB with 100 μg Rf ml^{-1}, incubating the culture at 30 °C and isolating an Rf^R clone after growth. Plasmid pB10 was then transferred from *Escherichia coli* K-12 (pB10) into these and all other Rf^R strains (all strains listed in Table 1, except strain H2) through conjugation and subsequent selection on LB-Rf (100 μg ml^{-1}) amended with the appropriate Tc and Sm concentrations. Strain H2 was obtained form creek sediment (Moscow, Idaho, USA) and pB10 was transferred into this host by conjugation with *E. coli* DH5α (Heuer *et al.*, 2007).

Stability experiments. Each strain harbouring pB10 was streaked from a −80 °C freezer stock on selective LB plates and incubated at 30 °C. For each strain, stability experiments were performed in triplicate, starting from three separate colonies which were each inoculated into 5 ml LB with the appropriate concentrations of Tc and Sm to select for pB10. After incubation for 24 h at 30 °C with shaking at 200 r.p.m., these cultures were washed to remove the antibiotics by spinning down 1 ml culture and resuspending the pellet in 1 ml saline. From these cell suspensions, 4.88 μl was transferred to 5 ml LB so that approximately 10 generations were obtained per 24 h growth cycle ($1:2^{10}$ dilution rate). These freshly inoculated cultures constituted time point zero. After they were diluted and plated onto LB plates, and an aliquot was stored at −80 °C, they were incubated for 24 h at 30 °C and 200 r.p.m. From then on, 4.88 μl of the full-grown cultures was transferred every 24 h to fresh 5 ml LB and incubated at 30 °C with shaking at 200 r.p.m. At certain time points, the cultures were diluted and plated onto LB plates. Determining the fraction of plasmid free cells in the population was done by replica-picking 50 randomly chosen colonies per culture from the LB plates onto LB-Tc, LB-Sm and LB plates, and scoring Tc^-Sm^- colonies.

Molecular confirmation of strain identity and plasmid carriage. Genomic DNA of constructed strains was obtained through

Table 1. Stability profile of plasmid pB10 in 19 bacterial strains

Organism	Strain*	Stability†	Origin
Alphaproteobacteria			
Ensifer adhaerens	S96 (+)	Low instability	De Gelder *et al.* (2005)
Ochrobactrum grignonense	S100 (+)	Stable	De Gelder *et al.* (2005)
Ochrobactrum tritici	S55 (−)	Low instability	De Gelder *et al.* (2005)
Sinorhizobium meliloti	RM1021 (−)	Stable	Diaz *et al.* (1994)
Betaproteobacteria			
Cupriavidus metallidurans	AE815 (−)	Stable	Top *et al.* (1994)
Delftia acidovorans	EEZ23 (−)	Stable	Ramos-Gonzalez *et al.* (1991)
Delftia acidovorans	C17 (−)	Stable	De Gelder *et al.* (2005)
Gammaproteobacteria			
Escherichia coli	K12 MG1655(−)	Sporadic loss	ATCC 47076
Pseudomonas aeruginosa	PAO1(−)	Stable	Stover *et al.* (2000)
Pseudomonas koreensis	R28 (+)	High instability	De Gelder *et al.* (2005)
Pseudomonas nitroreducens	R39 (+)	Stable	De Gelder *et al.* (2005)
Pseudomonas plecoglossicida	P18 (+)	Low instability	De Gelder *et al.* (2005)
Pseudomonas putida	UWC1 (−)	Stable	McClure *et al.* (1989)
Pseudomonas putida	S60 (+)	Stable	De Gelder *et al.* (2005)
Pseudomonas putida	S37 (−)	Sporadic loss	De Gelder *et al.* (2005)
Pseudomonas putida	H2 (−)	High instability	Heuer *et al.* (2007)
Pseudomonas veronii	S34 (+)	Low instability	De Gelder *et al.* (2005)
Stenotrophomonas maltophilia	P21 (−)	High instability	De Gelder *et al.* (2005)
Stenotrophomonas rhizophila	C20 (−)	Stable	De Gelder *et al.* (2005)

*The symbol in parentheses (+ or −) denotes the presence or absence of an indigenous plasmid in the strain. All strains indicated by + carried a plasmid that was larger than pB10 (64 kb) as observed by agarose gel electrophoresis. Some strains carried additional plasmids smaller than pB10: P18 (one; <5 kb) and S96 (three; 5–50 kb).

†Stable, no segregants detected during 210 generations; sporadic loss, few segregants but no clear sweep detected; high and low instability, see Figs 1 and 2, respectively.

an enzymic lysis (Qiagen, 2001), after which the DNA was purified with the MoBio UltraClean 15 kit (MoBio Laboratories), according to the manufacturer's specifications, and resuspended in 12 µl TE buffer. At certain steps in the experimental protocol, BOX PCR genomic fingerprinting (Rademaker *et al.*, 1997) was performed on genomic DNA extracts, to confirm the identity of (i) the segregants derived from the pB10::*rfp*-carrying sludge strains, (ii) the subsequently constructed pB10-carrying strains used in the stability experiments, and (iii) randomly chosen plasmid-containing and plasmid-free clones isolated during the stability experiments.

To confirm presence or absence of pB10 in strains, gel electrophoresis of plasmid DNA extracts (Kado & Liu, 1981; Top *et al.*, 1990) was performed and the presence or absence of a plasmid DNA band similar to that of a pB10-containing control strain was observed. This method was also used to examine which environmental strains harboured an indigenous plasmid. To determine whether these indigenous plasmids belonged to the IncP-1β group or whether other IncP-1β partitioning determinants were integrated into the chromosome, the presence of the IncP-1β-specific *trfA* fragment in these isolates was examined by PCR amplification (Götz *et al.*, 1996) using total genomic DNA as template. In addition, PCR was performed on selected strains (P21, H2, R28) to amplify an IncP-1β-specific *incC* fragment (primers: incC-F, 5′-AGGCACACGTCGAAGAACTC-3′; incC-R, 5′-AAAACACTGGTCACGGCAAT-3′) in a reaction containing 35 µl H_2O, 5 µl 10× buffer, 2 µl $MgCl_2$ (25 mM), 1 µl *Taq* DNA polymerase (5 U μl^{-1}) (all Promega), 5 µl dNTP (10 mM) and 1 µl each primer (100 mM), subjected to 94 °C for 1 min, 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s.

Competition experiments. To determine the plasmid cost in some strains, plasmid-free and plasmid-bearing ancestral strains were put in competition against each other in serial batch cultures. The cost (c) of the plasmid was determined as the difference in cell doublings between plasmid-free and plasmid-bearing cells, relative to the number of cell doublings of plasmid-free cells after 20 or 40 generations: $c=1-W=1-[\log_2(N_t^P/N_0^P)/\log_2(N_t^s/N_0^s)]$, where W is the relative fitness of the plasmid-containing (^{P}) versus the plasmid-free (^{s}) strain (Lenski *et al.*, 1991). Precultures of the competing strains were started separately by inoculating single colonies into 5 ml LB, containing Tc for the plasmid-bearing strain, and incubated at 30 °C with shaking at 200 r.p.m. for 24 h. To start serial batch competition experiments, these overnight cultures were washed and diluted 10 times, from which 24.6 µl of each competitor was inoculated into 5 ml LB in six replicates. From then on, 4.88 µl of the overnight-grown cultures was transferred every 24 h to fresh 5 ml LB and incubated at 30 °C with shaking at 200 r.p.m. Total and plasmid-bearing cell counts were determined on LB and LB-Tc plates after 0 and 2 days for H2, R28 and P21, and 0 and 4 days for S34, S55, S60, S100 and C17. Control cultures of strains H2, R28 and P21, starting with only plasmid-bearing cells, also in six replicates, were grown in parallel to correct for plasmid loss in the plasmid-bearing strain during the competition experiments. The fraction of new plasmid-free cells arising in the stability experiments after 2 days was used to correct the counts on LB agar plates after 2 days in the competition experiment.

Modelling the dynamics of plasmid segregants. Our approach to estimate the plasmid loss frequency, cost and transfer frequency

involved connecting three mathematical models (see Fig. 3) with time series data. First, a simple difference equation model was formulated that assumes that at any generation, the abundance of the plasmid-free cells (m) increases due to (1) plasmid loss of the wild-type cells (n) at a segregation frequency λ, and (2) growth of segregants by a factor of $2^{1+\sigma}$, where σ represents the selection coefficient or plasmid cost. This first model assumes that there is no conjugational transfer from plasmid-carrying cells to segregants. The solution to this segregation selection (SS) model was presented by us previously (De Gelder *et al.*, 2004). Joyce *et al.* (2005) showed that for statistical analysis purposes, the growth of the fraction of plasmid-free cells can be considered deterministic and unaffected by the daily bottlenecks. Assuming that every daily cycle (k) encompasses $l=10$ generations, then in what follows we measured time using generations, and note that, according to the experimental settings, data were only gathered at times (generations) $t=lk$.

The second model relaxes the assumption that no conjugational transfer occurs and assumes the conjugation rate to be dependent on the fraction of donor cells. This process is similar to a Michaelis–Menten enzymic reaction, where enzyme and substrate are the plasmid-carrying and plasmid-free cells, respectively (Andrup & Andersen, 1999). The Michaelis–Menten form can also be derived from first principles assuming that transfer works like a Holling type II functional response (Holling, 1965), whereby the amount of conjugations initially increases steeply as a function of the available recipient and donor cells, but subsequently levels off to a saturation limit. The model equations for this HT model are described in a parallel study (Ponciano *et al.*, 2007). This HT model incorporates two extra parameters: γ, the maximum conjugation frequency attained during a time interval, and θ, the fraction of plasmid-containing cells at which the conjugation frequency is half its maximum. A note of caution is needed. Simonsen *et al.* (1990) proposed a method to estimate the conjugation rate based on the differential equations models developed previously (Levin & Stewart, 1980; Levin *et al.*, 1979; Stewart & Levin, 1977). In these models, γ was defined as the fraction of the encounters between donor cells and plasmid-free cells that result in a plasmid transfer; the units of γ were ml per cell h^{-1}. In the difference equations used in our study, the conjugation frequency γ actually represents the maximum fraction of the encounters per unit of time between plasmid-free cells and plasmid-carrying cells that results in plasmid transfer (Ponciano *et al.*, 2007). Hence the discrete transfer frequency used here and the continuous transfer rate constant defined previously are different parameters, and their values cannot be compared.

The third model accounts for the possibility that during stability experiments, the plasmid loss dynamics are altered, for instance due to mutations in the host chromosome or plasmid that affect the plasmid cost. This third model, hereafter called the variable selection (VS) model, serves as an alternative hypothesis to the null hypothesis that during an entire plasmid stability experiment the growth of a proportion of plasmid-free cells follows essentially a deterministic pattern and that any stochastic component, besides random sampling, is negligible. The VS model adds just one extra parameter to the SS model: instead of using a fixed plasmid cost (σ), it assumes that at each time step, the plasmid cost (S) is drawn from a normal probability distribution with mean (σ) and variance (τ^2). This model is described in detail in Ponciano *et al.* (2007).

Statistical analyses. The statistical analysis of these models is described in detail by Ponciano *et al.* (2007). Briefly, since a randomly chosen set of a given number of clones (D) is tested at specific time points, each individual has a probability (x) of being a segregant and $1-x$ of being a plasmid-carrier. This defines a binomial sampling process with D trials and sampling probability equal to the model-predicted fraction of segregants x at time t. Thus, the three models proposed above do not exactly describe the dynamics of the plasmid-free cells, but this sampling process, described by Ponciano *et al.* (2007), accounts for the deviations of the observations from the predicted growth pattern. This statistical model allowed us to rigorously connect the data with the deterministic difference equations. In the case of the SS and HT models, this was done using the method of maximum-likelihood (Rice, 1995; De Gelder *et al.*, 2004), while accounting for the sampling process in the VS model was methodologically and conceptually more complicated. Details about these approaches can be found in Ponciano *et al.* (2007).

RESULTS

Variable plasmid stability in 19 hosts

To investigate whether the bacterial host affects the stability of a BHR plasmid, we monitored the change in the fraction of pB10-carrying clones in the absence of selection in populations of 19 different strains belonging to the *Alpha-*, *Beta-* or *Gammaproteobacteria* (Table 1). Plasmid stability was highly variable between the strains, ranging from no observed plasmid loss to rapid plasmid loss in about 80 generations. Stability was even variable within the same genus (e.g. *Pseudomonas* and *Stenotrophomonas*) or the same species (e.g. *Pseudomonas putida*) (Table 1). Although some strains harboured indigenous plasmids (Table 1), none showed PCR amplification of an IncP-1-specific *trfA* fragment. Moreover, there was no obvious correlation between the presence of an indigenous plasmid and the stability of pB10. These results together suggest that the indigenous plasmids were compatible with pB10 and not responsible for its observed instability in some of these hosts. Our findings thus indicate that the ability of a BHR plasmid like pB10 to be stably maintained in the absence of selection (i.e. an antibiotic) is highly variable between strains within its replication host range, and thus must be affected by as yet unknown host-specific traits.

In 10 strains, pB10 was considered stable, as no segregants at all were detected during 210 generations of growth (see Table 1, 'stable'). In all these cases, integration of pB10 into the chromosome was ruled out after observation of a pB10-specific plasmid band on an agarose gel. Two strains, *P. putida* S37 and *E. coli* K-12, showed a few segregants, but no clear sweeps within 210 and 330 generations, respectively (Table 1, 'sporadic loss'). The observation that pB10 was stable in 12 strains belonging to 9 different species shows that this IncP-1β plasmid can be stably maintained in most strains within its host range.

Three strains, *Pseudomonas koreensis* R28, *P. putida* H2 and *Stenotrophomonas maltophilia* P21, showed very high plasmid loss as the fraction of plasmid-containing cells dropped below 2 % after about 80 generations (Table 1, 'high instability'; Fig. 1). In these three strains, no indigenous IncP-1 *trfA* or *incC* PCR fragment was detected, suggesting that incompatibility through sharing similar replication or partitioning systems does not explain the low plasmid stability. The reproducibility between the three replicate stability tests for each strain was high. The pattern

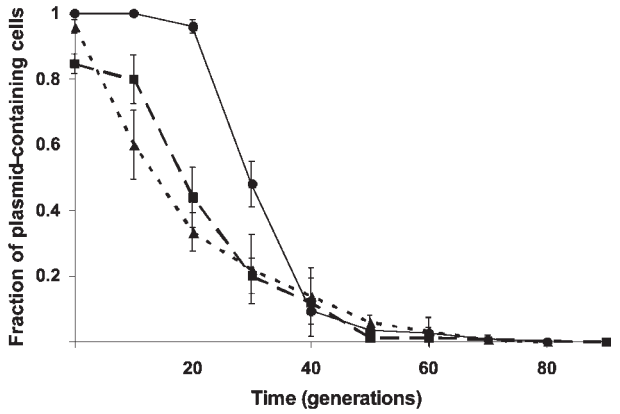

Fig. 1. High instability of plasmid pB10 in *P. koreensis* R28 (▲), *P. putida* H2 (■) and *S. maltophilia* P21 (●). Data points and error bars represent means ± SD of three replicates.

of decrease in the fraction of plasmid-containing cells was different between the three strains, which suggests that different underlying processes were responsible. These results demonstrate that even within the host range of the plasmid, some so-called 'unfavourable' strains do not retain the plasmid in the absence of selection.

Four other strains, *Pseudomonas veronii* S34, *Pseudomonas plecoglossicida* P18, *Ensifer adhaerens* S96 and *Ochrobactrum tritici* S55, showed much slower plasmid loss (Table 1, 'low instability'; Fig. 2). In contrast to the high reproducibility for the three strains that showed high instability (Fig. 1), there was moderate to substantial variation in the decrease of the plasmid-containing fraction between the three replicate stability tests for each of these four strains (Fig. 2). This high variability between replicates of such long-term experiments indicates that unknown factors influence the plasmid population dynamics over evolutionary time (see below). This observation of slow but unequivocal plasmid loss in the four strains examined points out the need to monitor plasmid stability for prolonged periods of time before drawing conclusions about the fate of a plasmid in bacterial populations.

Determination of the underlying cause of plasmid instability using mathematical models

Plasmid instability can be due to a combination of the following mechanisms: segregational plasmid loss, differential growth rates between the plasmid-free segregants formed and their plasmid-containing counterparts, and reinfection of segregants by conjugative plasmid transfer. To elucidate which of these mechanisms constituted the main underlying causes of plasmid instability in the different

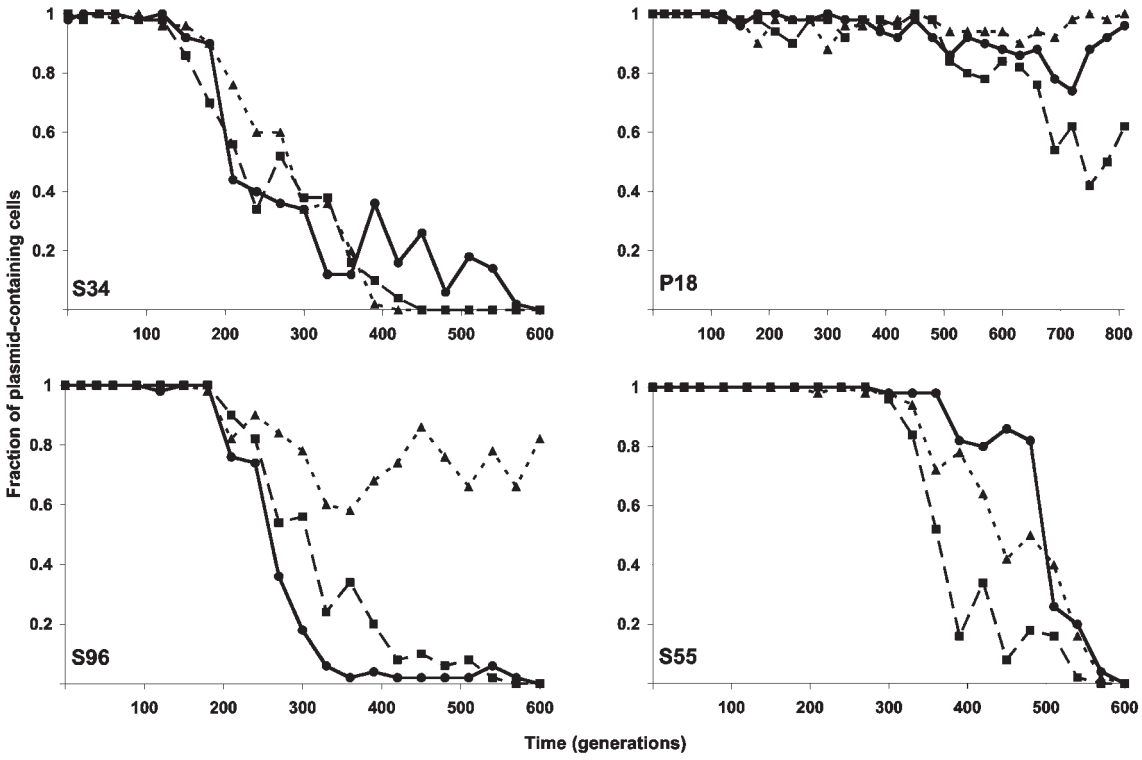

Fig. 2. Low instability of plasmid pB10 in *P. veronii* S34, *P. plecoglossicida* P18, *E. adhaerens* S96 and *O. tritici* S55 with high variability between the three replicates. For each strain, data from three independent replicate stability tests are presented. Note the different *x*-axis scale for P18.

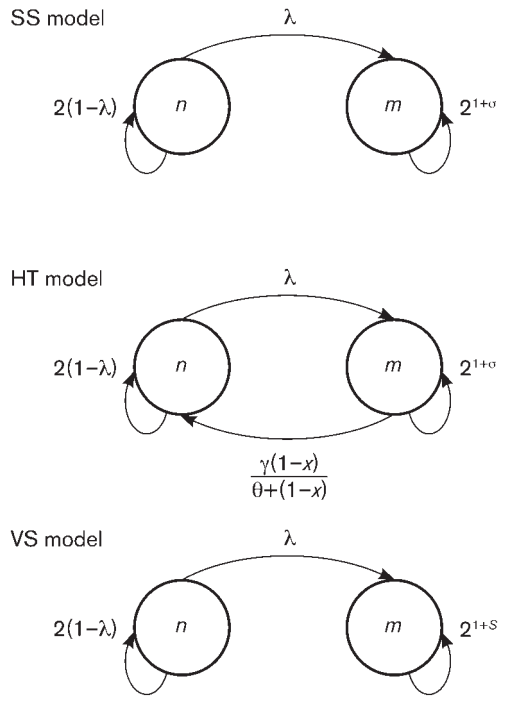

Fig. 3. Schematic representation of the three plasmid stability models and their parameters. In the SS model, the number (n) of plasmid-containing cells after one generation is twice the number of cells that did not lose the plasmid. λ denotes the segregation frequency at which a plasmid-containing cell gives rise to one plasmid-free daughter cell. The number of plasmid-free cells (m) increases from one generation to the next with factor $2^{1+\sigma}$, where σ is the selection coefficient or plasmid cost (constant). In the HT model, the plasmid reinfection depends on the fraction $(1-x)$ of available donors analogous to a Michaelis–Menten reaction, whereby γ is the maximum conjugation frequency and θ is the fraction of plasmid-bearing cells at which the conjugation frequency is half its maximum. The VS model is similar to the SS model, except that the selection coefficient (S) is now a normally distributed random variable, i.e. $S \sim N(\sigma,\tau^2)$. At every time point, a selection coefficient is drawn randomly from this distribution.

strains, three mathematical models were developed to approximate plasmid loss dynamics: the SS, HT and VS models. These models, which are described in detail in a parallel study (Ponciano *et al.*, 2007), are briefly summarized in Methods and conceptually represented in Fig. 3. For each of the seven strains that showed plasmid loss, the model that best captured the plasmid dynamics was first determined (see Table 1 in Ponciano *et al.*, 2007). The SS model, which considered only segregational plasmid loss and a fixed plasmid cost, provided a good fit to the data for two strains (R28 and H2), and more complex models did not fit significantly better, as confirmed by the absolute goodness of fit P-values.

Although conjugational transfer of IncP-1 plasmids in liquid cultures is thought to occur at rates too low to influence plasmid loss significantly (Bradley *et al.*, 1980; Gordon, 1992), we tested if incorporation of this mechanism in the model resulted in a significantly better fit to the data (HT model). This was the case for one strain, *S. maltophilia* P21 (Ponciano *et al.*, 2007). As shown in Fig. 1, the plasmid loss curve for this strain shows a longer lag phase than for strains H2 and R28, which may be explained by significant plasmid reinfection of rare segregants. For the four strains that showed much slower plasmid loss (S55, P18, S96 and S34), both deterministic models (SS and HT) failed to explain the plasmid loss dynamics. This was due to the erratic decrease of the pB10-containing fraction of the population and the high variability between replicates of the same strain. This variability was much higher than expected to arise from the observational error due to the sampling process alone. However, the non-deterministic VS model, which draws a selection coefficient from the distribution $S \sim N(\sigma,\tau^2)$ at each time step, provided a good fit for these datasets (Ponciano *et al.*, 2007). Overall, these results show that different models may be required to adequately capture the observed plasmid loss dynamics in different bacterial hosts.

Using the most appropriate model for each strain, the underlying parameters, i.e. segregation and transfer frequencies and plasmid cost, were estimated from the plasmid loss dynamics. Although pB10 was rapidly lost from the populations of the three strains, *P. koreensis* R28, *P. putida* H2 and *S. maltophilia* P21 (Fig. 1), different parameter estimates were obtained (Tables 2 and 3). First, in strains H2 and P21, the plasmid cost was estimated to be high, i.e. 14.6 and 58.9 %, respectively, whereas the segregation frequency estimates were low (Table 2). This implies that, after the slow formation of segregants, their numbers increased exponentially through their high growth advantage. In strain R28, however, the estimated plasmid cost was much lower (3.7 %), but the segregation frequency was very high (Table 2). This indicates that instability in host R28 was mostly due to rapid formation of segregants, which is in agreement with the observation that the plasmid-containing fraction decreased about 35 % during only 10 generations (Fig. 1). Second, out of these three strains, host P21 was the only strain for which the model including plasmid transfer (HT model) fit the data better than the SS model. Accordingly, the estimated transfer frequency (γ) was a significant value and much higher than that estimated for H2 and R28 (Table 3). To further illustrate the difference in the importance of conjugative transfer on overall plasmid stability in these strains, we compared the parameter estimates obtained with the SS model to those obtained with the HT model. Only for strain P21 were the estimates of segregation frequency and selection coefficient significantly different when calculated using the two different models (Table 3). These results strongly suggest that the involvement of plasmid reinfection through conjugative transfer in the stability dynamics was much greater for strain P21 than for strains H2 and R28. Together, these findings demonstrate that the main mechanisms responsible for rapid loss of a

Table 2. Estimates of segregation rate and plasmid cost for each strain showing plasmid loss

Plasmid cost was measured as the relative difference in fitness between the plasmid-bearing and plasmid-free strain. All experiments were performed in six replicates in LB broth. For strains H2, R28 and P21, control stability experiments were performed in six replicates to account for plasmid loss in the competition experiments. ND, Not determined.

Strain	Parameter estimates*		Experimentally determined plasmid cost (mean ± SD)
	Segregation frequency, λ	Selection coefficient or plasmid cost, σ (%) (95 % CI)†	
H2	1.91×10^{-9}	14.65 (9.56–16.37)	14.9 ± 2.2 %
R28	3.90×10^{-2}	3.69 (0.00–7.70)	5.4 ± 1.9 %
P21	1.43×10^{-5}	58.93 (41.13–110)	40.4 ± 5.0 %
P18	1.54×10^{-5}	1.11 (0.16–13.01)	ND
S34	2.41×10^{-5}	1.10 (0.16–11.58)	5.7 ± 0.8 %
S96	1.90×10^{-5}	1.07 (0.15–11.69)	ND
S55	1.32×10^{-5}	1.04 (0.14–11.91)	11.5 ± 0.6 %

*Parameter estimates as obtained by using the best fitting model (Ponciano *et al.*, 2007).
†For P21, H2 and R28, the 95 % confidence interval (CI) was calculated using parametric bootstrap; for P18, S34, S96 and S55, the 95 % credibility interval (CI) is the 2.5 and 97.5 percentiles of the estimated posterior distributions of the cost (Ponciano *et al.*, 2007). Credibility intervals are used in Bayesian statistics and are based on the posterior probability distribution for the parameters conditional on the data. A 95 % credibility interval means that there is a 95 % chance that the parameter lies within the credibility interval.

conjugative BHR plasmid can be different for different bacterial populations.

For the strains that showed slow plasmid loss, a fixed segregation frequency λ and a normal probability distribution of the VS coefficient S, with mean σ and variance τ^2, were obtained through parameter estimation using the VS model (Table 2). The parameter estimates for all four strains were very similar, $\lambda \cong 2 \times 10^{-5}$, $\sigma \cong 1.10\,\%$ and $\tau^2 \cong 16\,\%$ (Table 2). This indicates that the distribution from which plasmid costs were drawn at each time step was rather similar for these strains. Due to the complex nature of this model, a horizontal plasmid transfer component has not yet been included; this will be part of future modelling work. The small plasmid costs in combination with the low segregation frequencies explain the very long lag phases in the plasmid stability curves and the slower rates of decrease in the plasmid-containing fractions, compared to the plasmid dynamics of hosts H2, R28 and P21 (Fig. 2 versus Fig. 1).

Experimental determination of plasmid cost

The mathematically obtained plasmid cost estimates were compared to plasmid cost values calculated from competition experiments between plasmid-carrying and plasmid-free cells (Table 2). For the three highly unstable strains (H2, P21, R28), the empirically determined cost values were in good agreement with the parameter estimates. The experimental cost values for strains S34 and S55 were much higher than the mean (σ) of the estimated probability distribution S (see Fig. 3), but still fell within the 95 %

Table 3. Comparison of the parameter estimates by the SS and the HT models for strains in which pB10 is highly unstable

λ, Segregation frequency; σ, selection coefficient, γ, maximum conjugation frequency; θ, fraction of plasmid-bearing cells at which the conjugation frequency is half of its maximum.

Strain	SS model		HT model			
	λ	σ (%)	λ	σ (%)	γ	θ
H2	1.91×10^{-9}	14.65	2.48×10^{-9}	14.64	2.11×10^{-9}	1
R28	3.90×10^{-2}	3.69	3.90×10^{-2}	3.69	1.99×10^{-30}	1
P21	4.26×10^{-4}	29.2	1.43×10^{-5}	58.93	6.65×10^{-2}	2.20×10^{-1}

credible interval. It has to be pointed out that the plasmid cost values were empirically determined in the ancestral hosts, and thus are only representative of the plasmid cost at the beginning of the stability experiments (generation 0). They do not represent a mean of the distribution from which plasmid costs were drawn over the 600 generations of the experiment (σ is in the VS model). Overall, there was good agreement between estimated and empirically determined plasmid cost values, which further validates the mathematical models. In conclusion, these data reveal a high variation in cost of the same BHR plasmid in different strains.

DISCUSSION

To evaluate the persistence of a BHR plasmid in bacterial populations within its host range, we examined the stability of the BHR IncP-1β plasmid pB10 in 19 different strains that belong to the *Alpha-*, *Beta-* or *Gammaproteobacteria*. Plasmid stability was remarkably different between strains that belong to the same genus (e.g. *Pseudomonas* and *Stenotrophomonas*) or even the same species (e.g. *P. putida*) (Table 1). Although 10 of the 19 stains examined were obtained after intentionally screening for segregants from pB10::*rfp*-containing strains (De Gelder *et al.*, 2005), and may therefore be biased towards hosts in which pB10::*rfp* was rather unstable (some transconjugants did not yield segregants), there was still a high variability in the stability of pB10 among these environmental strains. Moreover, there was no correlation between the presence of an indigenous plasmid in these strains and the stability of pB10. These results demonstrate that within the host range of a BHR plasmid, 'unfavourable' hosts exist for which long-term persistence of the plasmid in that population is not guaranteed.

It has become clear that substantial genomic variation can exist between strains of the same species. High genomic diversity below the species and subspecies level has been revealed through the use of high-resolution molecular fingerprinting techniques (Schloter *et al.*, 2000). Indeed, high levels of genome divergence exist for strains with identical or very similar ($>99\,\%$) 16S rRNA gene sequences (Jaspers & Overmann, 2004; Thompson *et al.*, 2005). When sequencing three *E. coli* genomes, fewer than 40 % of all genes were common to all three (Welch *et al.*, 2002). Also, strain diversity at the level of gene regulation and genome usage has been identified as a significant contributor to within-species variation (King *et al.*, 2004). Therefore, when we consider the bacterial cell as the primary environment of the plasmid, the genotypic variation between strains of a bacterial species may encompass certain host factors that interact differently with plasmid functions, or on which plasmids can have a differential effect.

Mechanisms responsible for overall plasmid stability

Several mechanisms exist by which strain-specific traits can influence the overall stability of a BHR IncP-1β plasmid in the absence of selection for plasmid-encoded traits. First, as plasmid replication involves plasmid- and host-encoded factors (del Solar *et al.*, 1996; Espinosa *et al.*, 2000; Toukdarian, 2004), inefficient or impaired plasmid replication in certain strains can cause the copy number to decrease over a few cell divisions and thus increase the segregation frequency. We did not examine in this study whether or not plasmid replication was hampered in any of the strains.

Second, plasmid segregation may be elevated in some strains due to negative host effects on the active partitioning system, the only mechanism for stable inheritance that has been confirmed in IncP-1β plasmids. For all but one of our strains, we either estimated the segregation frequency to be low or never detected segregants at all, suggesting that plasmid partitioning was functioning well in these strains. However, the very rapid plasmid loss in *P. koreensis* R28, despite a moderate cost, was explained by a high segregation frequency (0.04 per generation; Fig. 1, Table 2). Recent experiments in our laboratory have shown that an IncP-1β mini-replicon pMS0506 (only containing replication and maintenance regions, *oriT* and a kanamycin resistance gene) was also very unstable in R28 (M. Sota and others, unpublished results). Therefore, it is likely that the high segregation frequency of pB10 in this host was caused by poor functioning of the partitioning or plasmid replication system. Poor partitioning could be due to poor interaction or increased undesirable competition between partitioning proteins such as KorB and/or IncC and host factors, or to decreased expression of the *incC* and/or *korB* genes. Also, improper activity or impaired interactions of the *kfrA* product could cause higher segregation, as it plays an important role in IncP-1β plasmid stability (Jagura-Burdzy & Thomas, 1992; Adamczyk *et al.*, 2006). Specific structural and physiological properties of the host cell may also influence partitioning, as the spatial distribution of plasmid copies in the cell could involve interactions with the components of the cell architecture (Gerdes *et al.*, 2000; Gordon *et al.*, 2004; Velmurugan *et al.*, 2003). It is unclear at this point which host–plasmid interactions involved in plasmid replication and maintenance could be differentially influenced in different hosts so as to explain the observed variation in stable plasmid inheritance.

Besides replication and segregational loss, a third factor that affects overall plasmid stability is the differential growth rate of plasmid-free and plasmid-containing cells, also referred to as plasmid cost or burden. If large enough, such a plasmid cost can result in strong selective sweeps of segregants in the absence of selection for the plasmid, as observed for P21 and H2 (Fig. 1) and to a lesser extent for S34 and S55 (Fig. 2). In some strains, however, a high cost was measured in spite of high plasmid stability, suggesting that the net plasmid loss rate was too low to generate significant numbers of segregants that can sweep through the population. Similarly, a high cost has been observed for RP4 in *E. coli* J53-1, although RP4-free clones were never

detected in J53-1 (RP4) populations propagated for over 1000 generations in the absence of selection (Dahlberg & Chao, 2003). In *P. putida* H2, the IncP-1α plasmid (RP4) and the IncP-1β minireplicon (pMS0506) were also shown to be very unstable (Heuer *et al.*, 2007; M. Sota & E. Top, unpublished results). This suggests that IncP-1 plasmids in general confer a high cost to H2 and that one or more of the replication and maintenance functions of these plasmids (the only backbone genes present on pMS0506) may be the underlying cause (M. Sota and others, unpublished results).

The cost of IncP-1 plasmids is thought to be minimized by an efficient regulation of plasmid copy number and gene expression through multiple plasmid-encoded repressors (Thomas, 2000), which would enable BHR plasmids to respond to variable concentrations of repressors in different hosts (Adamczyk & Jagura-Burdzy, 2003). Nevertheless, even with this flexible system of multiple plasmid-encoded regulators in place, a certain amount of resources will be withdrawn from the host metabolism for maintenance and expression of the foreign DNA, constituting the 'metabolic cost' of the plasmid on the host (Bentley & Kompala, 1990). Moreover, the antibiotic resistance genes present on plasmids are not subject to the overall plasmid gene expression controls and are often constitutively expressed (except for the *tet*-operon) (Poole, 2002; Ramos *et al.*, 2005). This might explain more moderate plasmid costs such as those observed in some strains in this study (Table 2). However, the higher plasmid cost observed in strains P21, S60 and H2 seems too high to simply represent metabolic cost and therefore could be due to a different negative effect of the plasmid on the cell, also called the 'plasmid-mediated interference cost' (Modi *et al.*, 1991).

Many negative effects of plasmid carriage on host physiology have been documented for vectors used for recombinant protein production, such as depletion of certain aminoacyl-tRNAs or amino acids, the enzymic activity or physical properties of plasmid proteins interfering with host-cell functioning and obstruction of proper exportation and/or localization of cellular proteins (Glick, 1995). Dramatic changes in the concentration of cellular enzymes involved in carbon, amino acid and nucleotide metabolism and translation have been associated with plasmid carriage, as well as a decrease in ribosome content and free 30S and 50S ribosome subunit pool fractions (Birnbaum & Bailey, 1991). Diaz Ricci & Hernández (2000) showed that the influence of plasmids on the host metabolism, measured as respiration rates, depended on the genetic background of the host. Also, stress induced by plasmid maintenance can often be related to the plasmid copy number (Bailey, 1993). When the plasmid copy number control is impaired due to host-specific interference, an elevated copy number might increase the amount of resources withdrawn from the host's metabolism. Although these observations were made for recombinant plasmids, which induce high levels of heterologous gene expression in the host cell, similar interactions between natural BHR plasmids and some hosts may occur. When host factors can affect the network of plasmid-encoded regulators, increased plasmid gene expression levels might lead to similar effects as observed with recombinant plasmids. Even at normal levels of IncP-1 plasmid gene expression, certain plasmid-encoded proteins may interfere with host-cell processes or even be toxic to the cell. To the best of our knowledge, little attention has been given to the nature of potential negative effects of naturally occurring plasmids on cell physiology or metabolism.

A fourth factor that may affect stability of conjugative plasmids is reuptake of the plasmid by segregants through conjugation with surrounding plasmid-containing cells. Validation of the different mathematical models and statistical analysis of the parameter estimates clearly suggested that for only one of the hosts, strain P21, including this horizontal gene transfer (HGT) process, explained the plasmid stability data better than segregational instability and plasmid cost alone. Separate empirical measurements of the transfer frequency (γ) for the different strains were not done for several reasons. First, as explained in Methods, this parameter is different from the transfer rate constant used in previously described models (Levin & Stewart, 1980; Levin *et al.*, 1979; Stewart & Levin, 1977), and thus the experimental method proposed by Simonsen *et al.* (1990) to empirically determine this parameter could not be used to measure γ. Second, this method assumes equal growth rates between donor and recipient, an assumption that would be violated for some strains due to the high plasmid cost. Moreover, rapid plasmid loss during the plasmid transfer experiment would also confound the calculations. Previous studies and our own data (unpublished) have repeatedly shown that IncP-1 plasmids transfer at very low frequencies in shaken or stirred liquid media compared to on solid surfaces (about 1000 times lower), presumably because of the short rigid IncP-1 pili that break due to friction (Bradley *et al.*, 1980). It is thus not so surprising that HGT did not significantly affect the plasmid dynamics in the other two strains that rapidly lost the plasmid (H2 and R28). Since the deterministic model with or without the inclusion of HGT did not adequately fit the plasmid stability data of the four hosts that showed slow plasmid loss (see below), and HGT has not yet been included in the complex VS model, conclusions about the role of HGT in plasmid persistence in these four strains cannot be drawn so far. Further research into the importance of HGT on plasmid persistence in continuously mixed as well as spatially structured environments is currently under way in our laboratory.

Evolutionary changes in host and plasmid may affect plasmid stability patterns during slow plasmid loss

The variation in plasmid loss between replicates of strains S34, P18, S55 and S96 (Fig. 2) was much higher than what was expected from variability due to sampling alone. Highly fluctuating frequencies of plasmid-containing cells over very short time spans have been documented before (Helling

et al., 1981; Modi & Adams, 1991). It was proposed that they were caused by mutations in the chromosome that were beneficial independently of the plasmid (Helling *et al.*, 1981). The fluctuations in the fraction of plasmid-free cells observed in our experiment are much smaller and mostly followed a general decrease in the plasmid-containing fraction. This suggests that general background mutations increasing the overall growth rate do not explain our observations. However, specific mutations that decrease the cost of the plasmid to the host can temporarily cause an increase or stabilization in the plasmid-containing population. As different mutations can arise at different time points in the three replicates, a variable plasmid cost throughout the experiment and substantial differences between replicates is not unexpected. During long-term growth of plasmid-carrying hosts in previous studies, chromosomal mutations that diminished the plasmid cost, or even enhanced host fitness only in the presence of the plasmid, have been documented (Bouma & Lenski, 1988; Modi & Adams, 1991). It is thus conceivable that over the course of 600 generations in our stability experiments, mutations arose that changed the plasmid cost relative to that in the ancestral host. The significantly better fit of the VS model over the SS model supports the hypothesis that the selection coefficient is variable between time points. However, the VS model cannot predict whether the plasmid cost decreased over time, as this would require the cost (S) to be an explicit function of time. In our model, S is normally distributed, with the same probability distribution at all time points. To estimate the parameters of such an extended model, a very high number of replicate stability tests would be needed for each strain. Another extension of the VS model that is needed, and will be part of future modelling work, is the inclusion of the conjugative plasmid transfer component. Nevertheless, the current data and available VS model clearly indicate that factors other than segregational loss and a fixed plasmid cost affect the stability dynamics over prolonged time periods, and a plausible candidate is the occurrence of mutations that affect plasmid cost over evolutionary time.

Conclusions

We have shown that some strains within the traditional host range of a BHR plasmid do not or poorly retain the plasmid in the absence of selection for known plasmid-encoded traits. As there was no correlation between the presence of an indigenous plasmid in these strains and the stability of pB10, we conclude that specific host–plasmid interactions must lie at the basis of this observed variation in plasmid stability. Plasmid-encoded proteins may affect normal host-cell functioning, or specific host factors may derail proper plasmid replication, partitioning or the control of plasmid gene expression. Our results thus suggest that stability and other characteristics of BHR plasmids have to be evaluated relative to their main environment, i.e. their bacterial hosts.

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