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METHODS

Plasmid incompatibility: more compatible than previously thought?

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It is generally accepted that plasmids containing the same origin of replication are incompatible. We have re-examined this concept in terms of the plasmid copy number, by introducing plasmids containing the same origin of replication and different antibiotic resistance genes into bacteria. By selecting for resistance to only one antibiotic, we were able to examine the persistence of plasmids carrying resistances to other antibiotics. We find that plasmids are not rapidly lost, but are able to persist in bacteria for multiple overnight growth cycles, with some dependence upon the nature of the antibiotic selected for. By carrying out the experiments with different origins of replication, we have been able to show that higher copy number leads to longer persistence, but even with low copy plasmids, persistence occurs to a significant degree. This observation holds significance for the field of protein engineering, as the presence of two or more plasmids within bacteria weakens, and confuses, the connection between screened phenotype and genotype, with the potential to wrongly assign specific phenotypes to incorrect genotypes.
Keywords: plasmid compatibility/origin of replication/ antibiotic resistance

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Introduction

Plasmids containing the same origin of replication are generally considered incompatible (Novick, 1987; Nordstrom and Austin, 1989; Sambrook *et al.*, 1989; Austin and Nordstrom, 1990), that is they cannot stably co-exist in a cell together. In this concept of incompatibility, it is thought that competition for replication factors leads to competition between plasmids, with those having growth advantages, such as faster replication (due to smaller size, for example), or less toxicity, rapidly outgrowing other plasmids in the cell. This is particularly important when the plasmid copy number is low. Given the many replication cycles occurring in a single overnight growth, even small differences in competitive advantage are thought to be able to lead to rapid dominance of a culture by a single plasmid. We have noted, however, the occasional appearance of bacterial clones containing more than one phagemid in phage display selection experiments, and recently exploited this observation to create a large antibody phage display library by intracellular recombination (Sblattero and Bradbury, 2000). When making this and subsequent libraries, we found that at least 18 different phagemids could infect a single bacteria, which after recombination could lead to the persistence of at least 40, and probably more than 200, different phagemids for at least two overnight growths. However, as novel phagemids are being continually generated during this recombination process, traditional Darwinian selection is unlikely to be able to operate, as no single phagemid persists long enough to be selected against. This is not to say that selection does not occur, as we have often noted the appearance and dominance of deleted forms that cannot undergo recombination if growth is continued for too long. These deleted clones are present in the initial library and are likely to be less toxic as they do not express antibody fragments, so possessing a selective advantage over all the other phagemids, which, whatever their sequence, express antibody fragments. This phenomenon of plasmid persistence has also led to a number of high-profile retractions, where phenotypes ascribed to specific plasmids were actually due to plasmid mixtures (Altamirano *et al.*, 2002; Zeytun *et al.*, 2004).

The frequency of double transformants is significantly higher than generally appreciated (Goldsmith *et al.*, in press). However, the persistence of plasmids after transformation has not been carefully studied. In order to characterize this phenomenon of plasmid persistence more carefully, and how it relates to plasmid copy number and recombinant DNA manipulations, we carried out experiments in which plasmids containing the same origin of replication and different antibiotic resistance genes were introduced simultaneously into bacteria by phagemid particle infection. By selecting on one of the antibiotics, and subsequently testing clones on the others, we were able to determine the percentage of bacteria containing more than one plasmid, and found that plasmid persistence is far more common than generally realized.

Results

Co-infection with three phagemids occurs easily

In order to determine the level of plasmid persistence, plasmids based on pBluescript (Stratagene, La Jolla, CA, USA) containing three antibiotic resistances (ampicillin, kanamycin and tetracycline) and two origins of replication (colE1 and pBR322) were used (Mayer, 1995). These are named A1, A2, K1, K2, T1 and T2 where A, K and T signify the resistance to ampicillin, kanamycin and tetracycline, respectively, and 1 indicates the pBluescript origin of replication (colE1 origin), and 2 is the pBR322 origin. The estimated copy numbers of these origins are 400–700 (Sambrook *et al.*, 1989; Lin-Chao

et al., 1992; Lee *et al.*, 2006b) and 15–75 (Lin-Chao and Bremer, 1986; Sambrook *et al.*, 1989; Atlung *et al.*, 1999; Lee *et al.*, 2006a, 2006b), for colE1 and pBR322, respectively.

Plasmid DNA was introduced into bacteria by infection using phagemid particles prepared using a recently described bacterial packaging cell system (Chasteen *et al.*, 2006). By providing all required filamentous phage packaging proteins in trans, this system is able to produce genetically pure phagemid particles that are uncontaminated with helper phage. By using infection, rather than transfection, to introduce phagemid DNA into bacteria, extremely high transduction frequencies can be obtained, allowing the accurate titration of functional phagemid DNA, and the transduction of equal ratios of different plasmids. Phagemid particles prepared using the plasmids described above and the M13cp helper plasmid (Chasteen *et al.*, 2006) were titered and phagemids containing the different antibiotic resistance genes were mixed at equal titers for each of the colE1 and pBR322 incompatibility groups. These two phagemid particle mixtures were infected into *Escherichia coli* (DH5αF′) at a multiplicity of infection of 20 : 1 and plated on agar plates containing ampicillin, tetracycline, kanamycin or no antibiotic (2XTY). These conditions are similar to those we previously used to enable the entry by multiple phagemids prepared using standard helper phage (Sblattero and Bradbury, 2000). Plating was only carried out on a single antibiotic, rather than on all three antibiotics simultaneously, to avoid interference between different resistances. We have found that if the multiplicity of infection is kept high, all bacteria become infected by all three phagemids simultaneously, since the numbers of colonies obtained on each antibiotic is identical to the number of colonies when plated on no antibiotic (Fig. 1). Although this indicates that each bacterium was infected by phagemids representing all three antibiotic resistances, it says nothing about the ability of these phagemids to persist in the bacteria.

Co-survival of plasmids after multiple overnight growth cycles

For each of the two combinations of plasmids, i.e. A1 + B1 + C1 (colE1 origin), or A2 + B2 + C2 (pBR322 origin), 384 colonies were picked from each of the three agar plates (ampicillin, tetracycline and kanamycin) and inoculated into liquid media containing the same antibiotic as the plate from which the colonies had been picked, and grown overnight. Bacteria from these plates were then inoculated into media containing each of the three different antibiotics (ampicillin, kanamycin and tetracycline) and grown overnight again. The following day the number of wells showing growth was counted. Growth was determined by dividing the absorbance (OD 550 nm) of the media after overnight growth by the absorbance of the starting inoculum, and assuming that an increase of more than 2.5-fold indicated growth. This process was repeated for an additional three days, with each selection plate replica inoculated (at a dilution of 1/100) into three sets of microtiter plates each containing one of the three different antibiotics. This experiment essentially involves multiple overnight growth cycles in one antibiotic with testing for resistance in other antibiotics after each overnight growth. The scheme for the whole experiment is illustrated for bacteria grown in media containing ampicillin (Fig. 2). As a result of the initial plating on agar plates, and the first overnight growth in liquid media, the point at which plasmid survival is first assessed is three days after the first plasmid transduction.

The results of survival over three further overnight growths are indicated in Fig. 3. For growth in each antibiotic, regrowth in the same antibiotic yielded 100% growth of all wells, as expected. With regard to the maintenance of plasmids that were not selected for, Fig. 3A shows the results for the high copy number plasmids. Persistence of plasmids carrying kanamycin or ampicillin for a total of five overnight growths was essentially 100%, whatever the initial antibiotic resistance selection, while the persistence of plasmids encoding tetracycline resistance was slightly reduced to 95% for three and four overnight growths, and approximately 80% by the fifth overnight growth. The situation with plasmids carrying the lower copy origin of replication (Fig. 3B) was clearly different, with some plasmids (kanamycin after selection in tetracycline) completely lost after five overnight growths. However, even though low copy plasmids were lost more rapidly in the absence of selective pressure than the high copy plasmids, the degree of persistence was still remarkable: over

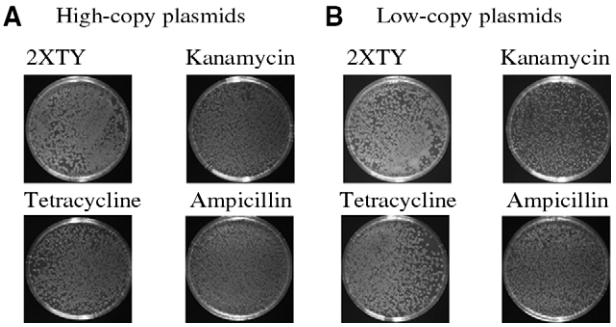

Fig. 1. Bacteria infected with the equimolar mixture of phagemids at a multiplicity of infection of 20 : 1 were diluted and plated on 2XTY, kanamycin, ampicillin or tetracycline plates. (**A**) Phagemids containing the colE1 origin of replication. (**B**) Plasmids containing the pBR322 origin of replication.

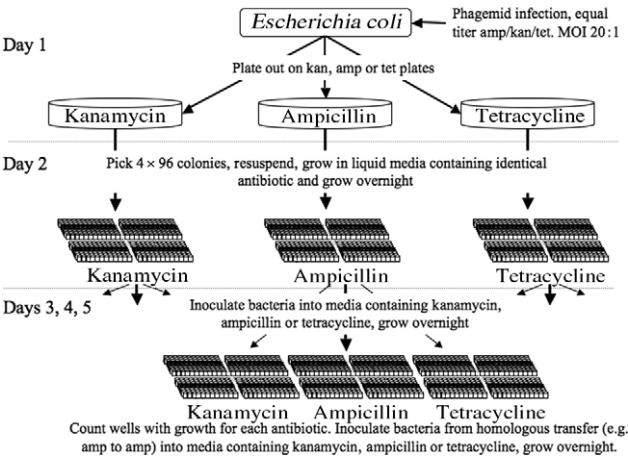

Fig. 2. Experimental scheme used to determine the plasmid persistence. After infection with a mixture of phagemids at a multiplicity of infection of 20 : 1, bacteria were plated out on agar plates containing kanamycin, ampicillin or tetracycline. For each antibiotic plate, 4 × 96 colonies were picked and resuspended in liquid media containing the same antibiotic. After overnight growth, 2 μl of each culture was transferred to 200 μl of medium containing each of the three different antibiotics in different plates. Growth in each of the antibiotics was assessed the following day. The procedure was repeated for two further days, always starting with innoculae from those plates maintained in the same antibiotic (bold arrows).

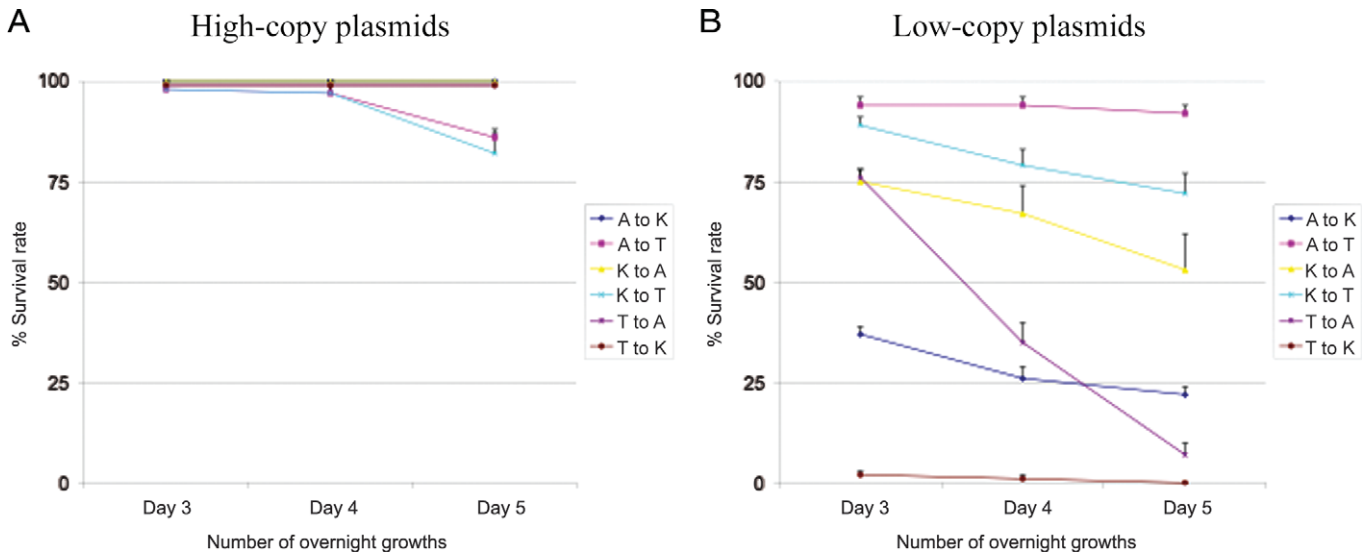

Fig. 3. Plasmid survival in bacteria after growth in liquid culture. The percentage of wells showing growth in each antibiotic after previous selective growth in a different antibiotic is shown for phagemids containing the colE1 origin (**A**) and the pBR322 origin (**B**) after three, four and five overnight growths. A, ampicillin; K, kanamycin; T, tetracycline. A to K indicates that bacteria were first selected in ampicillin for the number of overnight growths indicated and then tested for their ability to grow in kanamycin.

75% of bacteria for four of the six combinations maintain more than one plasmid in the absence of selective pressure for at least three overnight growths, and this is reduced to 50% for half the combinations after five overnight growths.

Percentage of bacteria carrying more than one plasmid

As the experiments above were carried out in liquid medium, in theory it should be sufficient for one bacterium to carry an additional persistent plasmid for the whole culture to be scored as positive after overnight growth. For this reason, rather than scoring growth in an 'all or nothing' fashion, we also examined the percentage of bacteria in a smaller set of 12 wells for each selection that carried antibiotic resistances carried by persistent plasmids which were not selected for. This was carried out after the third overnight growth, as in the experiments above. The number of bacteria in each well were titrated on plates containing ampicillin, kanamycin, tetracycline or no antibiotic whatsoever (to obtain the total number of bacteria), allowing us to calculate the total number of bacteria carrying plasmids with each antibiotic resistance. These were then expressed as a percentage of the total number of bacteria as shown in Fig. 4. Within the error limits, and as expected, the results show that all bacteria contained the antibiotic resistance for which the culture was selected for, and that for the high copy plasmids depending upon the antibiotic combinations examined, up to 100% (selection on kanamycin and testing on ampicillin) of the bacteria contained persistent plasmids. All the plasmids were persistent in at least 20% of bacteria and most were found in ~50%. In the case of the low copy plasmids, some combinations (e.g. selection on ampicillin, testing on tetracycline) were extremely persistent, while others, such as kanamycin resistance, were lost unless selected for. Still others remained persistent at relatively high levels.

Discussion

The results presented here show that different plasmids containing identical origins of replication can be stably maintained in bacteria for periods that are experimentally significant. While this appears to contradict the widely accepted doctrine of plasmid incompatibility (Nordstrom and Austin, 1989; Sambrook *et al.*, 1989), which is defined as the failure of two coresident plasmids to be stably inherited in the absence of external selection (Novick, 1987), it is clear that for most of the combinations tested, plasmid loss is eventually observed, even if it sometimes takes many days. The loss of such plasmids is thought to be caused by an inability to correct changes in the copy number of one of the plasmids arising from stochastically unequal replication or partitioning of individual plasmids within the plasmid pool, an effect which is aggravated if one of the plasmids has a growth advantage over the other, due to smaller size or lower toxicity, for example. While plasmids with low copy numbers are actively partitioned (Pogliano, 2002), those with high copy numbers, such as the ones used here, appear to be replicated at discrete sites and diffuse through the cytoplasm, undergoing passive partition between daughter cells (Pogliano *et al.*, 2001; Nordstrom and Gerdes 2003), relying on high numbers to ensure equal transmission to daughter cells.

A complementary way of considering plasmid incompatibility is within the context of the plasmid loss rate for bacteria containing single plasmids. This is described as the probability of forming a plasmidless cell per cell per cell generation, and for any one cell containing n plasmids can be calculated to be $(1/2)^{n-1}$ (Chiang and Bremer, 1988; Summers 1991). If a bacterium contains two different plasmids with identical origins, each population of plasmids will have a plasmid loss rate which is $(1/2)^{(n/2-1)}$ compared to $(1/2)^{n-1}$ for the same origin represented as a single plasmid. For bacteria containing three plasmids, as used here, the loss rate will be $(1/2)^{(n/3-1)}$. Application of these formulas to a copy number (45) at the center of the range of published estimates for pBR322 (15–75) gives predicted plasmid loss rates of 6×10^{-14} when present as a single plasmid, 3×10^{-7} when present as two plasmids and

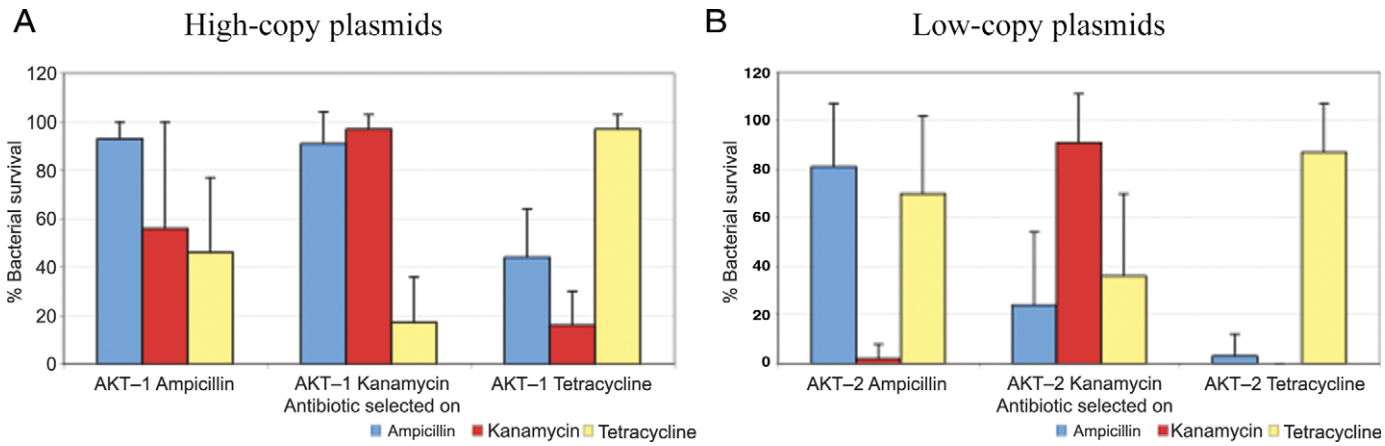

Fig. 4. Percentage of bacteria containing persistent plasmids after selection on a single antibiotic. After two overnight growths (one on agar, one in liquid) in each of the antibiotics, the number of bacteria able to grow on each of the individual antibiotics and 2XTY were assessed. The number growing in 2XTY was normalized to 100%, and growth on each of the antibiotics was normalized to this value. The figures shown are the means of 12 wells for each combination. AKT-1 Ampicillin indicates that the mixture of phagemids containing the colE1 origin were grown in ampicillin before being tested on each of the antibiotics indicated. AKT-2 refers to the mixture of phagemids containing the pBR322 origin.

6×10^{-5} when present as three plasmids, showing a loss rate which becomes significant as the number of different plasmids is increased and mirroring the results obtained here. In the case of pUC, even using the lowest copy number estimate of 400, the equivalent figures are 10^{-120}, 10^{-60} and 10^{-40}, each of which can be considered to be zero for practical purposes. However, these estimates are significantly altered by the distribution around the mean copy number. Plasmids with broad distributions will be far more unstable than those with tight distributions. This is especially true if the broad distribution leads to some dividing cells having copy numbers less than 20, and the loss of plasmids provides a growth advantage allowing plasmid-free cells to outgrow those with plasmids (Summers, 1991). The situation is additionally complicated by the presence of plasmid multimers, which tend to further increase plasmid loss (Summers and Sherratt, 1984).

Although these theoretical assessments provide some basis for understanding the results obtained here, the ability of modern cloning vectors containing different inserts to co-exist on experimental time scales has not been previously examined. This has become particularly important in directed evolution experiments where plasmids containing genes with small differences are generated by mutation, transformed and then screened for specific phenotypes. If bacteria selected on the basis of desired phenotypes contain more than one plasmid, and such plasmids can co-exist, the lack of a strict coupling between phenotype and genotype can create problems in downstream analysis. In the experiments reported here, we show that plasmids can co-exist without the need to apply any selection pressure for relatively long periods, and certainly within the time scale of many such evolutionary experiments. In agreement with the theoretical discussion above, this ability to persist appears to depend primarily upon the copy number of the co-existing plasmids. For a period of five overnight growths, equivalent to approximately 50 generations, we found significant plasmid persistence with the pUC origin. This is in contrast to the low copy number plasmids, in which plasmid loss was clearly present early on and continued with further growth. That said, even with the low copy plasmids, considerable persistence remained (Figs 3B and 4B) with some antibiotic combinations. The nature of the antibiotic resistance gene carried, and by extension any differing DNA, was also important, although far less so than the copy number. It is difficult to draw conclusions about which genes are preferentially maintained in the absence of selective pressure, since this also appears to be dependent upon the plasmid copy number.

In the experiments reported here, we used infection, rather than transfection, to transduce plasmids into *E. coli* because of the far higher efficiency of infection, which allowed us to identify and analyze a statistically significant collection of clones. We would argue that once within bacteria, the pattern of persistence will depend upon the nature of the plasmids present, rather than the manner in which they entered (infection or transfection), and this is confirmed by preliminary experiments we have carried out. Within this regard, although they did not examine persistence, it is significant that Goldsmith *et al.* also report that it is relatively straightforward to introduce multiple plasmids into single bacteria by transfection. We expect the long-term persistence of such different plasmids to mirror those described here.

The plasmid persistence documented here has been exploited to generate diversity in phage antibody libraries by site-specific recombination (Sblattero and Bradbury, 2000). When the methyl-directed mismatch repair system is inhibited, either genetically (e.g. MutSL) (Denamur *et al.*, 2000), pharmacologically (e.g. 2 aminopurine) (Matic *et al.*, 2003) or *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (Mergeay and Gerits, 1983) or by stress (Bjedov *et al.*, 2003), recombination between differing DNA substrates can occur *in vivo*. This has been carried out with plasmids containing both different (Gomez *et al.*, 2005) and identical origins of replication (Doherty *et al.*, 1983) to select for plasmids that confer survival. Given the results described here, *in vivo* recombination, akin to *in vitro* DNA shuffling, could probably be carried out using a library of error-prone PCR products cloned into a single phagemid and introduced at a high multiplicity of infection. Furthermore, it is possible that two plasmid systems in which compatible origins of replication are usually used, for example bacterial two hybrid (Karimova *et al.*, 1998; Hu *et al.*, 2000; Joung *et al.*, 2000;

Joung, 2001; Serebriiskii *et al.*, 2005) bacterial protein complementation assays (Pelletier *et al.*, 1998; Harris *et al.*, 1999; Mossner *et al.*, 2001; Wigley *et al.*, 2001; Galarneau *et al.*, 2002; Koch *et al.*, 2006), and two plasmid systems for the simultaneous expression of different proteins (Kholod and Mustelin, 2001; Skowronek and Kasprzak, 2002) or RNA (Gabriel and McClain, 2001), could conceivably be replaced by plasmids with identical origins, although further studies in these systems, perhaps using two different antibiotic resistances, will need to be carried out to determine the feasibility of this concept.

Materials and methods

Plasmid description

The plasmids selected for the experiment are described in detail by Mayer (1995). DH5αF′ (F′/*endA1 hsdR17* ($r^{K-}m^{K+}$) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1 D (lacZYA-argF)U169 deoR* (F80*dlacD(lacZ)M15*)) was used for all infection experiments.

Phagemid preparation

Chemically competent DH5αF′ was transformed with the m13cp plasmid and each of the pMPM series plasmids. Bacteria containing both plasmids were selected by growth on LB 2XTY agar plates containing chloramphenicol and ampicillin (50 μg/ml), kanamycin (50 μg/ml) or tetracycline (15 μg/ml). A single colony was picked from each plate and inoculated into 50 ml 2XTY containing ampicillin, kanamycin or tetracycline, and grown overnight at 30°C to produce phagemids. The phage particles were prepared by PEG precipitation and titrated. Each of the six phage titrations were plated on all three antibiotic plates to assure that they had only the corresponding resistance and were not contaminated.

Multiple infection

One milliliter of DH5αF′ at OD_{600} 0.5 were infected at 37°C for 45 min with a mix of the three phagemid particles carrying the same origin of replication (1 or 2). Phagemids were added to bacteria at a multiplicity of infection of 20 : 1. After the initial infection, an aliquot of the bacteria was plated onto four different plates, each containing either one of the three different antibiotics, or 2XTY alone (Fig. 1). Uninfected bacteria were also plated to check for contamination. After overnight growth at 37°C, four sets of 96 colonies were picked into 2XTY media containing the corresponding antibiotic and grown at 37°C overnight. The following (third) day, the overnight cultures were replica inoculated, at a dilution of 1/100, into media containing ampicillin, kanamycin or tetracycline and grown at 37°C for 17 h. The OD_{600} of the initial inoculum and the overnight culture were measured. Growth was determined to have occurred when the overnight OD_{600} was at least 2.5-fold greater than the starting inoculum. Usual values obtained were greater than six, with growth in tetracycline yielding lower increase. This selection procedure was repeated for two more days.

In order to determine the percentage plasmid survival in individual bacteria, after the second overnight growth in liquid media, bacteria from 12 wells for each mixture of phagemids, grown with each of the three selection antibiotics were titrated and plated out on agar plates containing each of the three different antibiotics as well as no antibiotic at all. The total number of bacteria was considered to be that growing on no antibiotics. The number of bacteria growing on other antibiotics were counted and normalized to the number growing on no antibiotics and expressed as a percentage.

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References

Altamirano,M.M., Blackburn,J.M., Aguayo,C. and Fersht,A.R. (2002) *Nature* **417**, 468.
Atlung,T., Christensen,B.B. and Hansen,F.G. (1999) *Plasmid* **41**, 110–119.
Austin,S. and Nordstrom,K. (1990) *Cell* **60**, 351–354.
Bjedov,I., Tenaillon,O., Gerard,B., Souza,V., Denamur,E., Radman,M., Taddei,F. and Matic,I. (2003) *Science* **300**, 1404–1409.
Chasteen,L., Ayriss,J., Pavlik,P. and Bradbury,A.R. (2006) *Nucleic Acids Res.* **34**, e145.
Chiang,C.S. and Bremer,H. (1988) *Plasmid* **20**, 207–220.
Denamur,E., *et al*. (2000) *Cell* **103**, 711–721.
Doherty,M.J., Morrison,P.T. and Kolodner,R. (1983) *J. Mol. Biol.* **167**, 539–560.
Gabriel,K. and McClain,W.H. (2001) *J. Mol. Biol.* **310**, 543–548.
Galarneau,A., Primeau,M., Trudeau,L.E. and Michnick,S.W. (2002) *Nat. Biotechnol.* **20**, 619–622.
Gomez,A., Galic,T., Mariet,J.F., Matic,I., Radman,M. and Petit,M.A. (2005) *Appl. Environ. Microbiol.* **71**, 7607–7609.
Harris,R.L., Sholl,K.A., Conrad,M.N., Dresser,M.E. and Silverman,P.M. (1999) *Mol. Microbiol.* **34**, 780–791.
Hu,J.C., Kornacker,M.G. and Hochschild,A. (2000) *Methods* **20**, 80–94.
Joung,J.K (2001) *J. Cell Biochem.* **37**(Suppl), 53–57.
Joung,J.K., Ramm,E.I. and Pabo,C.O. (2000) *Proc. Natl Acad. Sci. USA* **97**, 7382–7387.
Karimova,G., Pidoux,J. and Ullmann,A. Ladant (1998) *Proc. Natl Acad. Sci. USA* **95**, 5752–5756.
Kholod,N. and Mustelin,T. (2001) *Biotechniques* **31**, 322–323, 326–328.
Koch,H., Grafe,N., Schiess,R. and Pluckthun,A. (2006) *J. Mol. Biol.* **357**, 427–441.
Lee,C., Kim,J., Shin,S.G. and Hwang,S. (2006a) *J. Biotechnol.* **123**, 273–280.
Lee,C.L., Ow,D.S. and Oh,S.K. (2006b) Quantitative real-time polymerase chain reaction for determination of plasmid copy number in bacteria. *J Microbiol Methods* **65:** 258–267.
Lin-Chao,S. and Bremer,H. (1986) *Mol. Gen. Genet.* **203**, 143–149.
Lin-Chao,S., Chen,W.T. and Wong,T.T. (1992) *Mol. Microbiol.* **6**, 3385–3393.
Matic,I., Babic,A. and Radman,M. (2003) *J. Bacteriol.* **185**, 1459–1461.
Mayer,M.P (1995) *Gene* **163**, 41–46.
Mergeay,M. and Gerits,J. (1983) *J. Gen. Microbiol.* **129**, 321–335.
Mossner,E., Koch,H. and Pluckthun,A. (2001) *J. Mol. Biol.* **308**, 115–122.
Nordstrom,K. and Austin,S.J. (1989) *Annu. Rev. Genet.* **23**, 37–69.
Nordstrom,K. and Gerdes,K. (2003) *Plasmid* **50**, 95–101.
Novick,R.P (1987) *Microbiol. Rev.* **51**, 381–395.
Pelletier,J.N., Campbell-Valois,F.X. and Michnick,S.W. (1998) *Proc. Natl Acad. Sci. USA* **95**, 12141–12146.
Pogliano,J (2002) *Curr. Opin. Microbiol.* **5**, 586–590.
Pogliano,J., Ho,T.Q., Zhong,Z. and Helinski,D.R. (2001) *Proc. Natl Acad. Sci. USA* **98**, 4486–4491.
Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbour Laboratory Press, Woodbury, NY.
Sblattero,D. and Bradbury,A. (2000) *Nat. Biotechnol.* **18**, 75–80.
Serebriiskii,I.G., Fang,R., Latypova,E., Hopkins,R., Vinson,C., Joung,J.K. and Golemis,E.A. (2005) *Mol. Cell. Proteomics* **4**, 819–826.
Skowronek,K. and Kasprzak,A.A. (2002) *Anal. Biochem.* **300**, 185–191.
Summers,D.K. (1991) *Trends Biotechnol.* **9**, 273–278.
Summers,D.K. and Sherratt,D.J. (1984) *Cell* **36**, 1097–1103.
Wigley,W.C., Stidham,R.D., Smith,N.M., Hunt,J.F. and Thomas,P.J. (2001) *Nat. Biotechnol.* **19**, 131–136.
Zeytun,A., Jeromin,A., Scalettar,B.A., Waldo,G.S. and Bradbury,A.R. (2004) *Nat. Biotechnol.* **22**, 601.

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