The use of two-dimensional gradient plates to investigate the range of conditions under which conjugal plasmid transfer occurs

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Gel-stabilized two-dimensional gradient plates were used to study the effects of pH, salt concentration and temperature on the conjugal transfer of plasmid RP4 between strains of *Escherichia coli* and *Pseudomonas putida*. The combinations of pH and salt concentration that permitted conjugation were mapped as a two-dimensional growth area occupied by transconjugants following conjugation. This conjugation domain was less extensive than the areas that supported growth of the parental strains, and showed evidence for the interactive effects of pH and salt concentration in determination of conditions that permitted conjugation. The size and shape of the conjugation domain was influenced by time, temperature, the identities of the donor and recipient bacteria, and the combination of donor and recipient bacteria.

Keywords: RP4, plasmid transfer, gradient plates, conditions

INTRODUCTION

There has recently been increasing interest in the potential for genetic transfer between bacteria in the environment. This has been driven mainly by a need to predict the fate of genetically manipulated DNA sequences contained within bacteria that might be released into the environment either accidentally or by design.

Resistance plasmids have been widely used as models for the study of genetic transfer, owing to the ease with which their transfer can be detected. Thus a frequent approach to the determination of the environmental conditions that favour conjugation has been to study plasmid transfer in laboratory matings, and to investigate the effects on such matings of variations in physical and chemical parameters that are relevant to the environment (Kelly & Reaney, 1984; Rochelle *et al.*, 1989; Fernandez-Astorga *et al.*, 1992). Owing to the difficulties that would be involved in the investigation of large numbers of different combinations of parameter values, these studies have tended to examine the effects of varying only one parameter at a time, and have generally provided limited information on possible interactive effects between different variables. Another limitation of these studies has been their concentration on identifying optimal conditions for transfer rather than the total range of conditions over which transfer can occur.

The two-dimensional gradient plate system described by Wimpenny & Waters (1987) has been used previously to

study the interactive effects of various environmental variables on bacterial growth, and to determine the sets of such parameters that define the total growth 'domain' of the organism. In the present paper we have explored the adaptation of this technique to study the effects of environmental variables on plasmid transfer. For this purpose we have used the resistance plasmid RP4, which, though not a typical 'environmental' plasmid, has previously been used in investigations of genetic transfer between bacteria characteristic of natural communities, and has been shown to transfer to 83 % of Gram-negative bacteria isolated from soil (Kelly & Reaney, 1984; Rochelle *et al.*, 1989).

The gradient plate technique is shown to be very effective in defining the ranges of conditions under which conjugations occur, and the results emphasize the importance of the influence of the donor and recipient bacteria on the conditions under which conjugation occurs.

METHODS

Bacterial strains. *Escherichia coli* C600(RP4), *E. coli* C600 Rif^R, *Pseudomonas putida* KT2440(RP4), and *P. putida* KT2442 (Rif^R) were obtained from our laboratory collection. At 37 °C on gradient plate medium (GPM) RP4 was shown to transfer between the *E. coli* strains at a frequency of 1.5×10^{-3} per donor bacterium. To guard against culture variation during the course of the investigation, all strains were periodically reisolated from deep-frozen glycerol cultures.

Fig. 1. Typical profiles of pH (a) and salt (b) concentration across two-dimensional gradient plates. Error bars show 95% confidence limits based on eight independent gradients.

Growth media. Liquid cultures were grown in nutrient broth (Oxoid) with shaking at 30 °C. GPM contained (g l⁻¹ in distilled water): brain heart infusion (Difco) 37, yeast extract (Difco) 3, glucose (BDH) 3, Bacto agar (Difco) 15. Selective media consisted of nutrient agar (Oxoid) supplemented with kanamycin (50 µg ml⁻¹), rifampicin (100 µg ml⁻¹) or both these antibiotics.

Gradient plates. Two-dimensional gradient plates were poured in Sterilin 100 mm square Petri dishes using an adaptation of the Sybalski wedge plate technique (Sybalski & Bryson, 1952) as described by Wimpenny & Waters (1984). The plates comprised GPM with pH and NaCl gradients at right angles to one another. Typically, gradient plates were prepared in batches of no more than six at a time, and one plate from each batch was sacrificed for analysis of the gradient. This was done by taking 5 mm cores at 10 mm intervals from transects across the plate, melting each core into 10 ml deionized water, and measuring pH with a pH meter, or salt concentration with a Corning conductivity meter, model 200. Typical gradient profiles are shown in Fig. 1.

Inoculation of gradient plates. The standard method used for all experiments was as follows: overnight stationary phase broth cultures (typically containing approximately 4×10^9 viable bacteria ml⁻¹) of donor and recipient were mixed in equal volumes and the surface of a gradient plate was immediately flooded with an excess (about 2 ml) of this mixture. The plate was then tilted for 1 min to allow excess inoculum to drain into one corner, from where it was removed by pipette. The following three methods that were specifically designed to prevent contact between donor and recipient prior to inoculation were also tried: (a) separate consecutive inoculation of donor and recipient; (b) mixing of donor and recipient inoculum pools on the gradient surface; (c) inoculation of the recipient to a gradient plate, and of the donor to a square non-gradient plate, followed by velvet and block replication of the donor lawn onto

the recipient lawn. As all methods gave the same results, the standard method was adopted on the basis of its simplicity.

Mapping of conjugation domains. After incubation of the gradient plates, during which conjugation occurred, trans-conjugant bacteria were detected by replica plating (using the velvet and block technique) to 100 mm square plates of selective medium containing kanamycin and rifampicin that were incubated for 16–20 h at the temperature most suited to the recipient bacterium (30 °C for *P. putida* and 37 °C for *E. coli*). Trans-conjugants grew confluent over a defined area that was recorded by hand tracing.

Determination of conjugation frequencies. GPM containing appropriate additions to give particular pH/salt concentrations was poured into standard 80 mm diameter circular Petri dishes. Plates were inoculated with donor/recipient mixture (as described above) and incubated for 2 h [4 h in the case of *E. coli*(RP4) × *P. putida*] at 30 °C. Surface growth was removed by suspension in normal saline (5 ml), serially diluted and counted for viable cells by plating samples of 100 µl onto nutrient agar containing kanamycin alone (to select for donors), rifampicin alone (to select for recipients), and kanamycin plus rifampicin (to select for transconjugants). Mean counts of triplicate plates were used to calculate transconjugant frequencies, which were expressed per surviving donor cell. Quoted frequencies are means of two independent matings.

RESULTS

Growth domains of bacteria

Bacteria of two different genera, *E. coli* and *P. putida*, were chosen as the experimental organisms in order to allow a study of both inter-generic and intra-generic conjugations. The particular strains *E. coli* C600 and *P. putida* KT2440 were used because of their lack of restriction/modification systems, which might otherwise depress levels of transfer in inter-generic matings. Rif^R-derivatives of these two strains were employed as conjugative recipients.

In order to determine their growth domains on the pH/salt gradient plates, each of the above bacterial strains was inoculated as a lawn onto gradient plates, and grown for 24 h at 30 °C. The two *E. coli* strains showed similar growth domains, and Fig. 2(b) shows a result typical of either. The two *P. putida* strains also showed similar growth domains to each other as typified by Fig. 2(a). The *E. coli* strains showed more extensive growth domains than the *P. putida* strains, penetrating about 1 pH unit further into the acid end of the gradient, and extending further into the regions of high salt/high pH. Mixed cultures of *E. coli* and *P. putida* (as used below for conjugation experiments) grew over an area that corresponded to the single-culture growth area for *E. coli*.

Effect of time on conjugation domains

Conjugal transfer of plasmid RP4 was studied in intra- and inter-generic matings that involved *E. coli* and *P. putida* as donor or recipient. All matings were carried out on two-dimensional salt/pH gradients as described in the Methods section, and the distributions of donor, recipient and transconjugants were detected by replica plating to appropriate selective media. The distributions of all

Fig. 2. Growth areas of (a) *P. putida* and (b) *E. coli* on two-dimensional salt/pH gradient plates after incubation for 24 h at 30 °C.

parental strains (not shown) were found to coincide with their growth domains in pure cultures (Fig. 2), but with a scattering of single colonies outside the main distribution. These colonies must derive from bacteria which, although situated in the regions of the gradient that do not support proliferation, have remained viable and resumed growth after transfer to a non-gradient plate. In all crosses, transconjugants were detected within well-defined regions of the gradient as shown in Fig. 3. These zones possessed sharply defined edges, though often with a few outlying colonies at the alkaline edge that have been ignored in the analysis. The sizes of the transconjugant zones increased with conjugation time, but even after 24 h were very much smaller than the growth domains of the participating parents.

When transconjugant bacteria were isolated, cultured, and inoculated as lawns to the same type of gradient plate, they showed growth domains that were closely similar to those of the recipient parent. This shows that the transconjugant zones obtained during conjugation on gradient plates reflect the conditions that allow conjugation to occur, and not merely the conditions that allow growth of the transconjugants. These zones can therefore be considered as conjugation domains, and are

Fig. 3. Effect of time on area of conjugation at 30 °C. Each square represents a single salt/pH gradient plate. Rows: A, *P. putida*(RP4) × *P. putida*; B, *P. putida*(RP4) × *E. coli*; C, *E. coli*(RP4) × *E. coli*; D, *E. coli*(RP4) × *P. putida*.

clearly more limited than growth domains of the participating parental strains.

Most of the plates showed evidence for interactive effects between salt concentration and pH, most obviously a narrowing of the pH range at which conjugation occurs as the salt concentration increases.

The extent of the conjugation domain showed some relationship to the identity of the recipient species. After conjugation for 2–4 h with *E. coli* as recipient (with either donor), transconjugants were observed over comparatively large areas with extension into the 5–6% (w/v) region of the salt gradient. By contrast, when recipient and donor were both *P. putida*, early transconjugants were limited to a relatively small domain, and extended only to 3.5% (w/v) salt. In the case of a *P. putida* recipient with *E. coli* as donor, no transconjugants at all were observed in the first 4 h. After 6–24 h with *E. coli* as recipient, the conjugation domain frequently extended (by extrapolation) beyond the top of the salt gradient, whereas with *P. putida* as recipient the top of the gradient was never reached. When *E. coli* was recipient the conjugation domain also extended further into the acid end of the pH gradient than when *P. putida* was recipient.

Effect of temperature on conjugation domain

Effect of temperature on conjugation domain was investigated for all four mating pairs. Six hours was adopted as a standard period for conjugation as in all cases, except *E. coli*(RP4) × *P. putida*, there was little extension of the area of conjugation after this period, and extended incubation times may result in significant re-transfer of plasmid from

Fig. 4. Effect of temperature on area of conjugation. Each square represents a single salt/pH gradient plate. Rows: A, *P. putida*(RP4) $\times$ *P. putida*; B, *P. putida*(RP4) $\times$ *E. coli*; C, *E. coli*(RP4) $\times$ *E. coli*; D, *E. coli*(RP4) $\times$ *P. putida*.

transconjugant to recipient (see Discussion). Results for a range of five temperatures are shown in Fig. 4.

In the two matings with *P. putida* as donor, the results were similar. No conjugation was observed at 15 °C, and differences in the size and shapes of conjugation domains at 20, 25 and 30 °C are probably not significant. At 35 °C the *P. putida*(RP4) $\times$ *E. coli* mating showed greatly increased sensitivity to salt concentration at pH values lower than about 7.0. With *E. coli* as donor, conjugation was detected at all temperatures. For *E. coli*(RP4) $\times$ *E. coli* matings, the conjugation domain was most extensive between 20 and 30 °C, with extension across the entire salt gradient at 25 and 30 °C. For *E. coli*(RP4) $\times$ *P. putida* matings, the conjugation domain was consistently smallest at 30 °C (a surprising result because 30 °C is generally considered close to the optimum growth temperature of *P. putida*), and at 35 °C a sensitivity to salt at pH values below neutral was reflected in a conjugation domain (Fig. 4u) of very similar shape to that seen in the reverse cross (Fig. 4j). This shape was therefore characteristic of matings involving the two different species at 35 °C.

The size and shape of conjugation domains was consistent in replicate experiments. The conjugation domains seen in the 6 h results of Fig. 3 and the 30 °C results of Fig. 4 are duplicate conjugations carried out at different times, and represent the extremes of variation that we have observed. The apparent differences seen in these examples become smaller if allowances are made for variations in the gradients between duplicates. For example, the salt gradient extends down to 2.2% in Fig. 3(c), but to only 3.2% in Fig. 4(d); the lower salt concentration in Fig. 3(c) has thus allowed conjugation to spread to the upper limit of the pH gradient, which is not the case in the higher minimum salt conditions of Fig. 4(d).

Fig. 5. Frequency of plasmid transfer on a salt/pH gradient plate. The mating combination is *P. putida*(RP4) $\times$ *E. coli*. Areas of conjugation at 30 °C are shown after incubation times of 2 h (black), 4 h (stippled) and 8 h (hatched). Superimposed numbers show categories of plasmid transfer frequency obtained in separate plate matings under salt/pH conditions found at the corresponding positions on the gradient plate. Categories (expressed as transconjugants per 10^3 donors) are: 1, > 1.0 ; 2, 0.5–0.14; 3, 0.062–0.015; 4, < 0.01 .

Optimum conditions for conjugation

The domain in which conjugation was first detected (i.e. at 2 h for many matings) was found to correspond to the domain in which conjugation was most frequent (measured as number of transconjugants per surviving donor). This was determined by performing matings on a series of different non-gradient plates, each of which represented a particular salinity/pH value, and estimating transconjugants as a fraction of surviving donors after a conjugation period of 24 h. Fig. 5 shows the relative

frequencies of transconjugants obtained from a *P. putida*(RP4) $\times$ *E. coli* mating at 14 different salt/pH values. Categories of conjugation frequency (1–4) have been superimposed at the appropriate salt/pH points on a gradient showing the extents of the conjugation domain for this cross after 2, 6 and 8 h at 30 °C. The highest frequencies can be seen to coincide with the centre of the 2 h domain, and frequencies fall away as conjugation proceeds into the 6 and 8 h domains. Similar results were obtained for the other mating combinations at 30 °C.

DISCUSSION

Our results demonstrate that the two-dimensional gradient plate technique can be used successfully to determine the interactive effects of pairs of parameters on the occurrence of conjugal plasmid transfer. Use of a series of plates incubated at different temperatures enables temperature to be added as a third dimension, though not as a continuous variable. We elected to use pH and salt concentration as the variables in the gradient plates, but there appears to be no reason that the technique could not be used to investigate the synergistic effects of other environmental factors, such as concentration of total carbon or metal ions.

Results showed that in pH/salt gradients, the conditions over which conjugation occurs (the conjugation domains) are well defined and much more restricted than the growth domains of the donor and recipient parents. The pH range for all conjugations was broadest at low salt concentration and narrowed as salt concentration increased. It follows, therefore, that pH/salt synergism creates regions in which the proliferation of donor and recipient bacteria can occur, but not the conjugal transfer and establishment of plasmid RP4. At low salt concentrations these regions comprise the extremes of pH that allow growth of parental strains, but at high salt concentrations they may include the entire pH range that supports growth.

The effects of environmental parameters (including temperature, pH, nutrient concentration, salinity and metal ions) on plasmid transfer have been previously investigated by a number of other groups (Singleton & Anson, 1981; Gauthier *et al.*, 1985; Trevors & Oddie, 1986; Khalil & Gealt, 1987; Van Elsas *et al.*, 1987; Rochelle *et al.*, 1989; Fernandez-Astorga *et al.*, 1992). The approach of all these studies differed from that of the present paper in that they measured the frequency of plasmid transfer in response to variation of environmental factors. In contrast, the gradient plate technique does not yield frequency data, and we have used it to map the entire range of conditions over which transfer can occur, regardless of frequency. Additionally, we have shown that early transconjugants occur in the gradient sector that provides the best conditions for high transfer frequency, and, therefore, the distribution of early transconjugants identifies the domain that provides the optimum conditions for transfer. On the basis of our results it would be predicted that further restriction of the time allowed for

conjugation would produce a corresponding decrease in the conjugation domain, and thus define optimum transfer conditions more precisely.

Other studies have also differed from ours in their tendency to study the effects of particular variables in isolation, and not to address the question of possible interactive effects of different variables. In cases where synergy has been investigated, it has usually involved temperature as one of the variables, and has used a very limited number of combinations of variables (e.g. Rochelle *et al.*, 1989; Fernandez-Astorga *et al.*, 1992).

Although Rochelle *et al.* (1989) studied the transfer of mercury resistance plasmid pQM3 from *P. cepacia* to both *P. aeruginosa* and *P. fluorescens*, other workers have used only single combinations of donor/recipient, and generally the same species for both. For example, the detailed work of Fernandez-Astorga *et al.* (1992) investigated transfer of plasmids exclusively between strains of *E. coli*. This approach has tended to lead to the assignment of optimal transfer conditions to a particular plasmid on the basis of its transfer characteristics in a particular donor/recipient combination. Our results show that the conditions under which conjugation occurs can be strongly influenced by the nature of the donor/recipient combination. For example, plasmid RP4 transferred significantly at 15 °C when *E. coli* was donor (with either recipient) but not at all when *P. putida* was donor (with either recipient) (Fig. 4a, f). Also, at 30 °C transfer was detected after 2 h for all combinations except *E. coli*(RP4) $\times$ *P. putida*, where transfer was not detected until 6 h (Fig. 3m, n, p). Another effect of the donor/recipient combination is seen at 35 °C (Fig. 4j, u), where both inter-generic crosses produced irregular-shaped conjugation domains with very limited penetration into the salt gradient. In view of these findings it is apparent that caution should be exercised in the definition of optimum plasmid transfer rates on the basis of single donor/recipient combinations.

In the interpretation of results from inter-generic crosses it needs to be considered that later conjugation events may include plasmid transfers from transconjugants to recipients. Transconjugants are known to be effective conjugal donors and can transfer plasmids at significantly higher rates than the original donors (MacDonald *et al.*, 1992). Thus the large increase in conjugation domain that occurs between 6 and 24 h in the *E. coli* (donor) to *P. putida* mating (Fig. 3p, q) may result from an initial low-rate inter-generic transfer, followed by a higher rate of intra-generic transfer between *P. putida* transconjugants and recipients.

It is apparent from our work that the definition of the conditions that will permit the transfer of a particular bacterial plasmid in the environment is an extremely complex process. Factors to be taken into account include not only the identity of the plasmid concerned, but the interactions of relevant environmental factors and also the identity of donor and recipient organisms, together with interactive effects that occur between them.

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