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Most of us feel, from time to time, that other authors have not acknowledged the work of our own or other groups or have omitted to interpret important aspects of their own data. Perhaps we have observations that, although not sufficient to merit a full paper, add a further dimension to one published by others. In other instances we may have a useful bit of methodology that we would like to share.

The Editors hope that readers will take full advantage of this new section and use it to raise matters that hitherto have been confined to a limited audience.

John Freer
Editor-in-Chief

Meeting Commentary

Molecular Biology and Ecology of Gene Transfer and Propagation Promoted by Plasmids

An increasing amount of effort is being directed towards environmental microbiology. The application of molecular techniques to microbial community analysis has resulted in revelations about the microbial diversity in natural habitats. Traditional techniques for isolation and culturing have failed to reveal the presence of more than 90% of the soil microbial community. Many other areas of environmental microbiology are benefiting from the application of molecular methods, for example tracking of bacteria in soil, and the study of plasmid and phage ecology. However, ecologists need to fully understand gene function and expression if molecular microbial ecology is to be successful. The spread of genes in the environment is of great interest in the study of population genetics, bacterial survival and selection.

Bacterial plasmids play an important role in bacterial populations as agents which can maintain genes at high copy number and promote their own spread as well as the spread

of chromosomal genes between bacteria in a microcosm. To fully understand the role of plasmids in this respect requires cooperation between molecular biologists and microbial ecologists, two groups whose differences in interests and scientific language have often impeded the exchange of information in the past. Despite the very successful BAGECO meetings (on Bacterial Genetics and Ecology) organized in recent years there still seems to be a gulf. To help to bridge the gap we are trying to set up a Network to pull together people with these different interests. A first step in this process was a small meeting organized through the Juan March Foundation in Madrid, Spain which ran from 14–16 February 1994, under the same title as this short report. The purpose of this meeting was to bring together molecular biologists and microbial ecologists to discuss plasmid-mediated gene spread. We felt that it was important for molecular biologists to think about the relevance of their studies to significant ecological questions while for the ecologists it was necessary to learn about the details of the plasmids they are working with to provide a more rational basis for their investigations. This summary is to highlight some of the topics discussed, to make connections to related work which perhaps was not described at the meeting, and to pull out some general conclusions and ideas for the future. The topics are dealt with as a series of questions.

What determines the host range of a plasmid?

Although a plasmid does not have to possess a broad host-range of replication to be able to promote gene spread, a promiscuous replicon will increase the frequency with which a trait can establish itself in recipient bacteria once transfer has occurred. The factors which distinguish narrow and broad host-range replicons are slowly starting to emerge. Studies on prophage P1 (D. Chattoraj, Bethesda, USA) and broad host-range IncP plasmid RK2 (D.R. Helinski, UC San Diego, USA) which were described, illustrated how replication origins which are activated by binding of a Rep protein to repeated sequences (iterons) in the replication origin are dependent on efficient interaction with the host intracellular environment including host-encoded replication proteins such as DnaA. Therefore, factors affecting this interaction may be critical in determining in which host a plasmid can

replicate. The C-terminus of the replication initiator protein TrfA of RK2 is highly conserved among members of the IncP family (P.B. Thorsted, Birmingham, UK) and some alterations of these amino acids destroy TrfA activity while not affecting DNA binding (Helinski), suggesting that they are needed for other functions such as interaction with host proteins. Some mutational alterations in TrfA protein destroy activity in a species-dependent way, thus reducing the host-range of the plasmid. Thus the borderline between narrow or broad host-range may be very fine. This was illustrated also by studies on pPS10 from *Pseudomonas savastanoi* whose inability to replicate in *Escherichia coli* can be overcome by reducing the temperature to 25°C or by a minor change (Ala–Val) in the pPS10 replication protein (E. Fernandez-Tresguerres, Madrid, Spain). This observation fits with the point emphasized by J. Fry (Cardiff, UK), that plasmids in bacteria which survive in soil or water are adapted to much lower temperatures than 30°C or 37°C and that molecular studies should be designed to reveal their natural properties rather than ones in unnatural laboratory conditions. Host-range needs to be applied to a specific set of conditions which are relevant to the issue in question.

Two other types of replicon were described: the rolling circle replication pMV158 family which can replicate in both Gram-positive and

► GUIDELINES

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Gram-negative bacterial species (M. Espinosa, Madrid, Spain), and whose host-range has been recently extended to corynebacteria (J. Hochmannova, Prague, Czech Republic); and the theta replication family of pSM19035 which has a broad range within Gram-positive bacteria (J. Alonso, Berlin, Germany). Studies with the pMV158 Δ mob-derivative pLS1 are uncovering the way that plasmid gene expression signals are balanced to maintain a stringent control on the synthesis of the initiator of replication protein, RepB (G. del Solar, Madrid, Spain), and how these signals respond to stationary phase (M. Espinosa-Urgel, Madrid, Spain). This may provide a first step in opening up the area of plasmid survival in non-growing bacterial populations.

Are the plasmids studied so far typical of those present in natural environments?

A major aim of ecological studies on bacterial plasmids is to classify the plasmids in natural environments to understand their likely properties and to determine the structure of those populations. For example, it is important to establish whether particular traits have spread clonally on a single plasmid or have moved from one replicon to another by transposition or some other recombination event. In the context of the meeting it was also important to determine whether the systems which have been analysed at the molecular level are representative of the plasmids present in soil or water. Efficient means of identifying plasmids are needed. Conjugative or mobilizable plasmids can be isolated by adding a selectable recipient strain and looking for plasmids which can carry a phenotype such as mercury resistance into this strain (Fry). Alternatively, plasmids can be identified by their ability to mobilize IncQ plasmids between strains (A. Wilmotte, Mol, Belgium). However, these plasmids still need to be classified along with the other indigenous plasmids for which selectable markers have not been identified. One way is to use hybridization or PCR probes for well characterized plasmid groups. For the IncP plasmids a need for replication origin probes for both IncP α and β subgroups was recognized while PCR probes for coding regions of essential proteins were described by D. Van Elsas, (Wageningen, Netherlands) and C.M. Thomas, (Birmingham, UK). The latter were based on IncP α / β sequence comparisons of highly conserved regions in both the *trfA* and *korA* genes. Another way is to isolate replicons from indigenous plasmids and to use these as probes for grouping the rest of the plasmids.

Of the plasmids so far described from the ecological studies, the IncP plasmids have received most attention and have been found in many species. They have been found during analysis of bacteria isolated from any depths of lake sediments (R. Pickup, Windermere, UK), from river epilithon (Fry) and soil (Wilmotte)

although some of the soil isolates have a narrower host range which could be due to genetic isolation and mutational drift, which is consistent with point mutations being able to reduce host-range without inactivating the replication system. The well studied IncP plasmids are typical of many new isolates in having a broad host-range: many plasmids from epilithon bacteria have an even broader host-range than well studied IncP plasmids. IncP β probes suggest that these may be related to R751 and others of this group to varying extents. The well established relationship of IncP plasmids to other plasmids from soil bacteria such as *Agrobacterium tumefaciens* and *A. rhizogenes* which has been demonstrated by sequence studies (F. de la Cruz, Santander, Spain and E. Lanka, Berlin, Germany) was extended to *Rhizobium leguminosarum* in which the first new plasmid replicon to be isolated has sequence similar to *Agrobacterium* Ti and Ri plasmids (S. Turner, York, UK) as well as to IncP plasmids in the *incC korB* genes.

What is required for full plasmid stability?

Considerable effort has been devoted to analysing those additional functions which reduce the probability of plasmid-free segregant bacteria from arising within populations of plasmid-positive bacteria. The mathematical analysis of this plasmid instability was discussed (K. Nordstrom, Uppsala, Sweden) to appreciate how variations in plasmid copy number per cell, multimerization (recombination between plasmids to give molecules containing tandemly repeated copies of the plasmid genome, thus reducing the number of separate plasmid molecules per cell) and the difference in growth rate of plasmid-positive and -negative bacteria can influence the apparent rate of plasmid loss. Mechanisms which ensure at least random segregation by resolution of multimers were discussed briefly but much more attention was devoted to various mechanisms for killing plasmid-free segregant bacteria by activating lethal gene products through degradation of an antidote protein (the *cad* system; M. Couturier, Gent, Belgium) or antisense repressor RNA (the *bok/sok* system; K. Gerdes, Odense, Denmark). Of particular interest were the *parD/E* genes of RK2 (Helinski) and *parD* system of R1 (R. Diaz-Orejas, Madrid, Spain) which appear to be cryptic, that is not normally functional. Changes in copy number however can activate these systems raising the possibility that some growth conditions or physiological states may unmask these genes and render them advantageous. True partitioning systems which are thought to provide a meiotic apparatus are still poorly understood despite recent progress in dissecting the plasmid-encoded proteins required and the *ais*-acting sequences in the plasmid on which these proteins act (R. Jensen, Odense, Denmark). A full understanding of the connection between external factors, physiological conditions, and plasmid stability

will require much work, concentrating on host-encoded as well as plasmid-encoded functions.

What influences plasmid transfer frequency?

Molecular studies on conjugative transfer of IncP plasmids have defined the genes required for both mating pair formation and initiation of rolling circle replication during transfer (Lanka). The gene sequences reveal similarities to the transfer apparatus of many other conjugative plasmids like the IncW group (de la Cruz) as well as having many similarities to the *vir* system of *Agrobacterium* Ti plasmids. Biochemical analyses have been instrumental in assigning functions to proteins acting at the *oriT* nicking stage (Lanka). Biochemical characterization of other conjugative stages awaits the development of a suitable *in vitro* replication system. Although this biochemical understanding thus has wide applicability, there still seem to be few clues about the factors which trigger IncP or IncW transfer. The discovery of control circuits which limit IncP transfer gene expression (Thomas) and which may provide a switch between genes for vegetative replication and conjugative transfer (G. Jagura-Burdzy, Birmingham, UK) hint at control of transfer in response to copy number, host physiology or growth phase. However, these could simply be a means of ensuring efficient production of the transfer apparatus without over-burdening the host with excess gene expression. A major area for the future is therefore to discover what controls transfer itself and why there is such a complex control of transfer gene expression.

The IncP plasmids, which exhibit transfer proficiency for all plasmid-positive bacteria differ from F-like plasmids which appear normally to shut off transfer gene expression so that conjugation only occurs after a random derepression event followed by exponential spread through the population of potential recipients. P-like and F-like plasmids differ also in pilus type. While the long flexible pili of F-like plasmids appear to promote transfer in the gut, only plasmids with short, rigid pili (P-like) seem to do well in soil when direct comparisons have been carried out (K. Smalla, Braunschweig, Germany). The role of pili is still not understood fully and indeed bacterium-plant mating does not appear to need a visible pilus to initiate the mating bridge formation, even though the apparatus is clearly related to that which promotes interbacterial mating.

Successful mating pair formation is not necessarily sufficient for DNA transfer and establishment in a recipient cell. For bacterium-plant cell mating many stages can be limiting: from recognition of the signal emitted by plant wound tissue which switches on *vir* gene expression, to integration of the transferred DNA once it has entered the recipient's nucleus (B. Hohn, Basel, Switzerland). For interbacterial matings restriction imposes a major barrier which can

be overcome in a number of ways. The best understood of these are saturation of the recipient restriction system by multiple rounds of transfer and delivery of an antirestriction gene whose product blocks the action of type I restriction endonucleases (B. Wilkins, Leicester, UK). Mutational elimination of target sites can also allow evasion of restriction barriers and analysis of IncP *tra* gene sequences suggests that its deficiency in certain restriction sites is consistent with a history of repeated transfer between the restriction enzyme-rich strains of *Pseudomonas aeruginosa*.

How do we study bacteria in 'natural environments'?

A major question which needs to be faced is how do we design and conduct experiments which will tell us about bacterial behaviour outside the laboratory. We first need to understand bacterial distribution and behaviour in natural environments. rRNA probes labelled with fluorescent or light-emitting tags (E.M.H. Wellington, Warwick, UK; S. Molin, Copenhagen, Denmark; and A.J. Palomares, Seville, Spain) allow differentiation between different species, and the intensity of fluorescent signal can give an estimate of growth rate. In the mouse gut, *E. coli* appears to grow at a rate which is too high to maintain a steady state (Molin). How this paradox can be resolved is not clear. In soil, bacteria are not spread evenly but become concentrated in small microcolonies which give locally high bacterial concentration (Wellington), thus increasing the frequency of genetic exchange above the level expected for an even distribution of similar bacterial counts through the same volume. Real environments are not uniform in space or time. In soil, the rich exudates produced by plant roots stimulate genetic exchange between bacteria, as do pulses of nutrients (Van Elsas), so that exchange in bacterial populations close to sources of these materials are favoured. Mixed populations also influence behaviour: well studied IncP plasmids appear to have a reduced ability to mate with recipients in a complex mixed population derived from epilithon when compared to newly isolated promiscuous plasmids from epilithon bacteria (Fry). A good way of studying bacterial behaviour during growth on surfaces and in biofilms has yet to be developed. Experiments can be performed on surfaces such as stone, glass or metal but analysing which layer is behaving in what way is more difficult. Ways of analysing the response of bacterial gene expression during growth on surfaces remains to be determined.

How do plasmids influence bacterial behaviour?

Despite the assertion that the presence of plasmids does not significantly influence bacterial behaviour (Molin), there seem to be a number of ways in which plasmids affect evolution of bacterial populations. Firstly,

plasmids can alter their host, not only by constituting a slight additional metabolic burden but by pleiotropic effects on the cell surface affecting such factors as phage sensitivity and virulence. The presence of IncP plasmids appears to reduce the *P. aeruginosa* virulence in a mouse model system, providing a possible explanation for why, in the absence of antibiotic selection, bacteria carrying RP4 can be displaced by plasmid-negative bacteria, as for example in the burns unit of the hospital where they were first isolated. Secondly, they can carry selectively advantageous genes such as antibiotic or heavy metal resistance in a multicopy situation. This not only alters phenotype by increasing resistance gene dosage which normally results in increased resistance, but also effectively produces gene duplication which increases the potential for mutational change without destruction of the primary advantageous phenotype. Those changes which give a marginal advantage will be selected under appropriate conditions and will allow the accumulation of point mutations which in combination produce a major change in resistance phenotype. Changes in resistance level are likely to occur through a series of very small steps rather than major jumps (F. Baquero, Madrid, Spain) and the selective advantage of each step can be dissected by site-directed mutagenesis. Plasmids may also promote the combination of single changes at a higher rate than double mutation but this has not been investigated. Some plasmids actually encode UV-repair, recombination and integral type functions whose expression appears to be coordinated with their replication. It was suggested that these may serve to repair and promote new combinations of plasmid-encoded genes (P.A. Battaglia, Rome, Italy). In addition we need to consider transposition as a major form of evolution – not only the movement of DNA from one location to another but also the way that transposons can be generated from combinations of transposition functions, phenotypic markers and regulatory elements. The potential for resistance mechanisms to develop to almost any antibiotic was illustrated with respect to vancomycin resistance in *Enterococcus* (P. Courvalin, Paris, France). This antibiotic binds an essential peptide component of cell wall biosynthesis rather than an enzyme and thus it was expected that the target would not be altered by a simple mutation. Resistance has arisen however by a new enzyme replacing D-alanine by D-lactate in the peptide, thus decreasing vancomycin binding. Since D-lactate is removed during cell wall assembly, this change does not reduce cell viability.

Can we influence plasmid survival in natural populations?

Given the worries about spread of recombinant plasmids it is important to consider ways of minimizing spread of such plasmids or the hosts that carry them. One well established

principle is to engineer a lethal function into the organism so that its job is done, for example in the degradation of an environmental pollutant, or in the case of a plasmid, its transfer to a new host can be inhibited by the plasmid carrying a gene which is lethal if not suppressed by a repressor encoded in the initial host bacterium. This principle was illustrated by the use of the colicin E3 gene and the immunity gene which regulates it (E. Diaz, Braunschweig, Germany). Since many of the applications of bacteria released into the environment will be to degrade xenobiotics such as phenol, toluene and chlorinated benzene derivatives, the way that bacteria sense these compounds and regulate gene expression in response to them is important. One feature which needs to be accommodated is the crosstalk between different regulatory systems (V. de Lorenzo, Madrid, Spain) since this can result in elevated levels of expression when the upstream activator regions are manipulated. This appears to be due to the increased activation by regulatory proteins bound at distant locations which can activate the $E\sigma^{54}$ RNA polymerase

The meeting played an important part in focusing attention on the issues where the interests of ecologists and molecular biologists meet. For the ecologists many issues came down to the best way of gaining knowledge of and access to the tools developed by the molecular biologists. For the molecular biologists the key questions appear to be how representative the systems they study are to plasmids in the environment and whether the bacterial hosts they use and the experimental conditions employed are likely to give a true picture of plasmid behaviour in natural environments. Overall these three days were successful in stimulating interactions between the two groups and laying the foundations for future meetings.

Comments relating to the issues raised in this report would be welcomed by the Editors.

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