Instituto Juan March
de Estudios e Investigaciones

23 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

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

Organized by

C. M. Thomas, E. M. H. Wellington, M. Espinosa
and R. Díaz Orejas

J.C. Alonso
F. Baquero
P.A. Battaglia
P. Courvalin
F. de la Cruz
D.K. Chattoraj
E. Díaz
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M. Espinosa
J. C. Fry
K. Gerdes
D.R. Helinski
B. Hohn
E. Lanka
V. de Lorenzo
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PROGRAMME

MOLECULAR BIOLOGY AND ECOLOGY OF GENE TRANSFER AND PROPAGATION PROMOTED BY PLASMIDS

MONDAY, February 14th

Registration.

I. Conjugation and Plasmid Transfer
Chairperson: K. Nordström

D.R. Helinski- Replication and Stable Maintenance Systems of Broad-Host-Range Plasmids.

F. de la Cruz- Overview on Bacterial Conjugation: Similarities and Differences Among Bacterial DNA Transfer Systems.

E. Lanka - IncP-Plasmid-Mediated Bacterial Conjugation: Functional Analogy to the Ti Plasmid T-DNA Transfer from Agrobacteria to Plant Cells.

B. Hohn - *Agrobacterium Tumefaciens* Transfers Single-Stranded T-DNA Into the Plant Cell Nucleus.

II. Replication and Maintenance
Chairperson: D.R. Helinski

M. Espinosa - Rolling Circle-Replicating Promiscuous Plasmids of the pMV158 Family.

J.C. Alonso - Maintenance Systems of the Gram-Positive Broad-Host-Range Plasmid pSM19035.

D.K.Chattoraj- Role of Iteron Sequences in Initiation and Control of P1 Plasmid DNA Replication.

R.Díaz Orejas- Regulation of ParD, a Killer Stability System of Plasmid R1.

M.Couturier - The *ccd* Killer System of Plasmid F.

K. Gerdes - Antisense RNA Regulated Programmed Cell Death.

C.M. Thomas - Molecular Genetics of Broad Host Range IncP Plasmids.

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TUESDAY, February 15th

III. Plasmid Transfer
Chairperson: B. Hohn

G. Jagura-Burdzy - Coordination of Promiscuous Plasmid Replication and Transfer: Regulatory Protein KorA of Broad-Host-Range Plasmid RK2 Acts Both Positively and Negatively at the Divergent *trfA/trbA* Promoter Region.

B. Wilkins - Mechanisms Allowing Plasmids to Evade Restriction Barriers to Transfer.

E.M.H. Wellington - Evironmental Factors Affecting Plasmid and Gene Transfer in Filamentous Soil Bacteria.

J.C. Fry - Genetic Transfer in Water.

R.W. Pickup - Distribution of Bacteria and Their Plasmids in Deep Freshwater Sediments.

J.D.van Elsas- The Occurrence and Role of Selftransmissible Plasmids in Soil and Rhizosphere.

A. Wilmotte - Exogenous Isolation of Conjugative Plasmids from Environmental Sources.

IV. Selected Talks from Posters
Chairperson: K. Gerdes

M.E.Fernández-Tresguerres - The Replication Protein of the Replicon pPS10 is a Host Range Factor.

M. Llosa - The-Amino Terminal Region of R388 TrwC Has *ori*T-specific Endonuclease Activity and Catalyzes Conjugation-Independent, Site-Specific Recombination at *ori*T.

R.B. Jensen - Plasmid Stabilization by the *par*A System of Plasmid R1.

C. Llanes - Characterization and Molecular Analysis of the Broad-Host-Range RepA/C Replicon.

S.L. Turner - Cloning and Sequence Analysis of Plasmid Origin (*ori*) Sequences from *Rhizobium Leguminosarum*.

E. Mellado - Determination of the Basic Replicon of the *Chromohalobacter marismortui* ATCC 17056 Plasmid pCM.

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A.J. Palomares - Tracking Recombinant Organisms in the Environment: Use of Eucaryotic Luciferase Genes for Detection of Genetically Engineered Bacteria.

W. Vahjen - The Soil Insect *Folsomia candida* Mediates a Highly Efficient Interspecies Gene Transfer of Recombinant Plasmids by Transformation.

K. Nordström - Quantitative Aspects of Plasmid Behaviour.

WEDNESDAY, February 16th

V. Clinical and Environmental Plasmid-Mediated Gene Transfer
Chairperson: C.M. Thomas

F. Baquero - Population Selective Amplification (PSA) and the Evolution of Plasmid-Mediated β-Lactamases.

P. Courvalin - Plasmid/Transposon-Mediated Spread of Glycopeptide Resistance.

P.A. Battaglia - The pR Plasmid as a Tool to Investigate the Molecular Basis of the Hospital Infections.

VI. Molecular Ecology
Chairperson: M. Espinosa

S. Molin - Detection of Microorganisms and Monitoring of Their Metabolic Activity in Natural Environments.

E. Díaz - A Novel System to Explore the Ecological/ Evolutionary Role of Barriers to Gene Dispersal in Bacteria.

V.de Lorenzo - Microbial Sensing of Aromatic Pollutants: Specificity and Cross-Talk Among Catabolic Promoters of *Pseudomonas*.

Summing up:
C.M. Thomas and E.M.H. Wellington

Bringing the Molecular Biology to the Environment (or Vice Versa): Plasmids as Tools.

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INTRODUCTION

R. Díaz Orejas and M. Espinosa

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Plasmids are extrachromosomal genetic elements that replicate autonomously within their hosts. These elements have been isolated from eukaryotic and prokaryotic cells, and have helped to basic and applied research mainly on three areas: Environment, Molecular Biology, and Biotechnology.

From the environmental point of view, one key feature of plasmids is their ability to colonize new hosts by various mechanisms of genetic exchange. In addition, plasmids are able to pick information from the host chromosome or from other plasmids and to transfer it to new hosts. This transfer of genes leads to a lateral gene spread in the environment, in which plasmids play a fundamental role. The ability of plasmids to colonize different hosts can be achieved by conjugation, mobilization, or transformation from the donor to the recipient cell through complex mechanisms not yet well understood. As a consequence of this lateral gene spread, plasmids contribute to the genetic traits of the host by supplying it with new information. This, in turn, may favour the adaptation and survival of the host in a changing environment. Several relevant genetic features are plasmid-borne, as it is the case of some resistances to antibiotic and to heavy metals. In addition, the ability to degrade many organic pollutants by soil bacteria is due to plasmid-encoded gene products. Thus, the knowledge gained in plasmid biology have helped to understand the appearance of new antibiotic-resistance traits and their spread among unrelated bacteria, as well as the design of new bacterial strains to be used as detoxifying organisms.

Many basic Molecular Biology studies have been performed with plasmids for various reasons: i) their relatively small size allows their manipulations; ii) since plasmids are no essential, their loss do not lead to undesireable consequences to the host, and iii) interactions between plasmid- and host-machinery can be studied. The use of plasmids has contributed to the understanding of fundamental processes as DNA replication and its control, analysis of transcription complexes, and influence of local DNA deformations on replication and on gene expression. Generally speaking, basic studies on plasmids have provided powerful tools to study DNA structure and function as well as DNA-protein interactions.

In addition to the above, plasmids have been widely used as natural genetic vehicles to obtain a wide collection of artificial vectors. They can be designed to construct useful bacterial strains containing and expressing new genetic information of biotechnological relevance. As a consequence, bacterial strains harbouring recombinant

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plasmids are being employed in a variety of products designed for human and animal welfare. However, the release to the environment of bacterial species genetically manipulated, and its potential impact on the environment, has posed a problem to the scientific community as it has raised some concerns in the society. This implies that detailed anaylises are needed, both in pure cultures and in more natural ecosystems, on several plasmid functions related to their lateral spread. Some of these studies should be focussed on: i) mechanisms of transfer among hosts; ii) features of colonization and establishment in new hosts; iii) mechanisms of replication and its control; iv) plasmid-genetic exchanges, and v) self-contained bacterial strains and plasmids.

Up to now, Plasmid Biology has been approached either from molecular biologists or from microbial ecologists. These groups have had little communication among them. However, the time has come in which a confluence between molecular and ecological approaches will enrich this field with the posing of new questions and the sharing of methods. All these reasons led to the co-ordinators of this Workshop to organize, for the first time, a meeting between leading scientists working on molecular, ecological and clinical aspects of plasmid biology. We were fortunate that this initiative was taken by the Juan March Foundation, which provided the economic and logistic support to organize this Workshop on "Molecular Biology and Ecology of gene transfer and propagation promoted by plasmids". We believe that this first gathering will be fundamental in promoting similar kind of initiatives in a near future that will lead to the confluence between Molecular Biology and Microbial Ecology in a new and fast developing discipline, the Molecular Ecology.

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I. Conjugation and Plasmid Transfer

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REPLICATION AND STABLE MAINTENANCE SYSTEMS OF BROAD HOST RANGE PLASMIDS

D.R. Helinski, Department of Biology and Center for Molecular Genetics, University of California, La Jolla, California

It is clear that in addition to the regulation of initiation of plasmid replication a number of other factors, plasmid and bacterial host encoded, contribute to the stable maintenance of broad host range plasmid elements. Our own laboratory has concentrated our research efforts on the broad host range plasmid RK2 which is capable of replication and stable maintenance in a wide-range of Gram-negative bacteria. We have focused largely on the biochemical properties of the RK2 replication initiation protein TrfA, the iteron-containing origin of replication of this plasmid, and a 3.2kb region of RK2 that provides stable maintenance in all Gram-negative bacteria tested to date. The TrfA protein binds specifically to the iterons at the origin of replication and likely forms a higher order nucleoprotein complex involving host replication proteins. This suggests that TrfA has the exceptional ability of interacting with host proteins from a diverse set of bacteria. Structure-function analysis of TrfA has identified regions of this protein that may be especially important in its interactions with host proteins. The additional ability of the TrfA protein to couple two RK2 origins of replication may play a role in the regulation of copy-number of this plasmid.

Although the TrfA protein and the RK2 origin of replication are sufficient for the replication of this plasmid in different Gram-negative bacteria, stable maintenance of the plasmid requires other plasmid encoded sequences. A major contributor to stable maintenance is a region of the plasmid (3.2 kb in size) that specifies a multimer-resolution system, a toxin-antitoxin system capable of killing cells under conditions of plasmid loss and possibly other stabilizing functions. The toxin-antitoxin system, encoded by a 0.7 kb segment, in itself is capable of providing a substantial level of stabilization in several different and diverse Gram-negative bacteria. However, full stabilization generally requires the entire 3.2 kb segment under conditions where, at least in E. coli, significant amounts of multimers are not formed, suggesting additional stabilizing functions for this segment.

The plasmid encoded elements described above clearly play an important role in the propagation of this plasmid amongst distantly related bacteria. When one examines the intact RK2 plasmid, it is just as clear that other elements specified by this plasmid, in addition to transfer functions, also are very likely to play a role in the high degree of promiscuity exhibited by this extra chromosomal element. The continued analysis of the RK2 class of plasmids in addition to other plasmids, both narrow and broad host range, will undoubtedly reveal several major processes that account for a naturally occurring plasmid occupying either a specific bacterial niche or being capable of stable maintenance in a range of bacterial hosts.

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Overview on bacterial conjugation: similarities and differences among bacterial DNA transfer systems

Fernando de la Cruz

Departamento de Biología Molecular, Universidad de Cantabria, Santander, SPAIN.

Conjugation is an important evolutionary tool for bacteria. The genetic information for conjugal DNA transfer is coded for by a variety of plasmids in Gram-negative bacteria. By means of conjugation, plasmids spread among bacterial populations, and colonize very distantly related genera. Plasmids carry also accessory genes, including transposons, which help bacterial (and plasmid!) survival. Not only can conjugative plasmids transfer their own DNA, they can mobilize other plasmids, which can be transferred in the presence of the conjugative plasmid in the same bacterial cell. Finally, they can also mobilize chromosomal genes, producing merodiploid cells and thus contributing also to chromosomal evolution. A lot of information concerning conjugation has recently been compiled in an excellent collection of reviews edited by D.B. Clewell (1993).

The prototype conjugative system is that of plasmid F (TRA_F). It occupies 34 kb of DNA and contains more than 30 genes involved in one or more of a discrete number of steps in which the conjugation process has been dissected:

1. Pilus and mating pair formation
2. Stabilization of mating pairs
3. Processing of DNA for conjugation
4. Surface exclusion
5. Regulation

Broadly speaking, there are two general classes of transfer systems in Gram-negative bacteria. Those that conjugate well in liquid media (which produce flexible pili), and those which can only transfer in solid media (which produce rigid pili). There are also pilus-specific phages, and transfer systems can be classified according to phage-sensitivity.

Among conjugative plasmids, the genetic organization and complete nucleotide sequence of only three groups is presently known: TRA_F, TRA_P and TRA_W. Several others have been partially sequenced: TRA_I, TRA_N and TRA_{Ti}. They all show some commonalities, which probably reflect that they share a common molecular mechanism for the DNA transfer process. In TRA_W, the tra genes are nicely organized in two regions: PIL_W contains ten genes (trwDEFGHIJKLM) involved in pilus synthesis, export and assembly, and MOB_W contains three genes (trwABC) involved in DNA processing for conjugation (Llosa et al., 1994). Other TRA systems have no so clear cut organization; TRA_P has also two separate regions, Tra2 being equivalent to PIL_W, while Tra1 is far more complicated than MOB_W. TRA_F contains a single operon with over thirty genes, in which the genes equivalent to MOB_W occupy the distal part

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Several of the proteins involved in the transfer process are significantly related in their primary sequence. These similarities appear even more fundamental if we consider that they are also conserved in two other systems. The vir system responsible for the transfer of the T-DNA from Agrobacterium tumefaciens to plant cells, and the ptl system, implicated in the export of the pertussis protein toxin from Bordetella pertussis (S. Bolland, M. Llosa and F. de la Cruz, unpublished).

Although usually different transfer systems can coexist in a cell, they interact with each other, in processes that have not been studied in much depth. One important interaction is called "fertility inhibition". Related transfer systems can have regulatory elements that can act upon the heterologous systems. This is the well-known case of plasmid F, being inhibited in its transfer ability by fi^+ plasmids. But also much less related transfer systems like TRA_N, TRA_P and TRA_W affect each other fertility.

Another kind of interaction is the mobilization of one oriT by a heterologous TRA system. These interactions appear to be very plasmid-specific for different conjugative plasmids, but are far more relaxed for mobilization of a special set of plasmids that are mobilizable but non-conjugative. Mobilizable plasmids are small, and can also be grouped in several related groups, although a systematic analysis of the molecular species, as carried out for conjugative plasmids, is not yet available. Several of them have been completely sequenced and there is genetic information about the genes involved in the mobilization process. Perhaps the best known examples are RSF1010, pSC101, ColEI and CloDF13. They are mobilized by the different conjugative plasmids widely variable efficiencies, and the reasons for it are beginning to be understood (Cabezón et al., 1994).

In order to have a coherent picture of the molecular basis of conjugation in the bacterial kingdom we need to know more about the diversity of the TRA systems, and the specialization that a given system has undergone to adapt to a particular ecosystem.

REFERENCES

Clewell, D.B. (Ed.) Bacterial conjugation. Plenum Press, New York (1993).

Llosa, M., Bolland, S. and de la Cruz, F. (1994) Genetic organization of the conjugal DNA processing region of the IncW plasmid R388. J. Mol. Biol. 235:in press.

Cabezón, E., Lanka, E. and de la Cruz, F. (1994) Requirements for mobilization of plasmids RSF1010 and ColEI by the IncW plasmid R388: trwB and RP4 traG are interchangeable. J. Bacteriol.: submitted.

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IncP-Plasmid-Mediated Bacterial Conjugation: Functional Analogy to the Ti Plasmid T-DNA Transfer from Agrobacteria to Plant Cells

Erich Lanka, Werner Pansegrau, Dietmar Balzer, Peter Scheiffele, Monika Lessl, Jana Haase and Rudi Lurz
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Plasmids of the incompatibility group P (IncP) are large self-transmissible broad host range plasmids carrying multiple antibiotic resistance determinants. The prototype IncP plasmid RP4 (60,099 bp) can replicate in diverse Gram-negative organisms. RP4 was originally discovered in a *Pseudomonas* clinical isolate. However, the host range identified by conjugative transfer ability is considerably greater than the range of vegetative replication. Suitable shuttle vector constructions carrying the IncP transfer system allow tracing of DNA transfer from Gram-negative organisms to Gram-positive bacteria and to yeasts.

Functions responsible for conjugative transfer of RP4 are encoded by two distinct regions on the genome known as Tra1 and Tra2. Essential transfer functions - the core of the Tra regions - were identified and evaluated by genetic analyses (1, 2, 3, 4). Transfer functions are classified in two groups: the *D*NA *t*ransfer and *r*eplication system (Dtr) and the *m*ating *p*air *f*ormation apparatus (Mpf) involved in bringing the donor and recipient cell into an intimate contact during conjugation. Dtr genes exclusively map in Tra1. This region consists of at least three operons and includes the origin of transfer (*oriT*) an intergenic region between the leader and the relaxase operon. Dtr functions participate in relaxosome formation - the initiation complex of the transfer replication process (5). Relaxosomal components TraJ and TraK are *oriT* specific binding proteins each of them recognizing sequences/structures adjacent to the cleavage site (*nic*). Complex formation of TraJ - *oriT* with TraI - the relaxase - eventually results in cleavage at the nick site (*nic*) of *oriT* by transesterification between the deoxycytidyl residue at *nic* and the hydroxyl of tyrosine 22 of TraI. Generation of the single plasmid strand destined to be transferred may resemble a rolling circle type of mechanism (6). DNA transfer is thought to occur *via* TraI-piloted single-stranded intermediates that maybe coated by the TraC protein (7). The DNA-TraC complex is likely to be transported through a channel or pore at the mating bridge between donor and recipient cell. Formation of the channel might depend on gene products of the Mpf system. Mpf genes map in Tra2 (*trbB, C, D, E, F, G, H, I, J, K* and *L*) and include *traF* of Tra1 (4).

Requirements of the Mpf apparatus were defined by two phenotypic observations: (i) donor specific phage propagation, *i.e.* IncP plasmid dependent phage growth, (ii) mobilization of the non-conjugative transmissible IncQ plasmid RSF1010. RSF1010 specifies its own relaxosome, however, relies on Mpf components for its transfer of conjugative plasmids. RSF1010 mobilization by RP4 in addition to Mpf requires *traG* of Tra1. The Mpf system (11 plasmid-encoded components) is believed to function in several processes: (i) pilus assembly and erection, (ii) contact formation and maintenance during conjugation between donor and recipient *via* recognition of the recipient by the pilus and subsequent pilus retraction (iii) DNA export into the recipient and (iv) phage DNA import by injection. The proposed phage receptor complex and part of the DNA transport machinery may consist of

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the same basic Mpf components (8). Most of the Mpf components are membrane or membrane-associated proteins as can be concluded from their primary structures.

The hypothetical relationship between agrobacteria-plant interaction and bacterial conjugation was originally proposed by Stachel and Zambryski in 1986 (9). *Agrobacterium tumefaciens* is a plant pathogen which genetically transforms plant cells. Introduction of a distinct DNA element, the T-DNA, into the plant's genome results in tumor formation. The T-DNA is located on a large extrachromosomal element, named Ti (*tumor-inducing*) plasmid (~200 kb). This plasmid encompasses *vir*ulence regions (Vir) providing most of the products involved in T-DNA movement. The *vir* loci comprise six complementation groups (~30 kb). Some of them are essential (VirA, B, D, G) or enhance the efficiency (VirD, E) of plant transformation. The T-DNA is flanked by 25 bp direct repeats, the right and left border sequences (TBS). At these *cis*-acting sites single-stranded scission occurs initiating the generation of the T-DNA strand to be transferred. Stachel and Zambryski compared the DNA transmission systems in terms of functional analogies: (i) *oriT* and TBS, (ii) respective DNA processing functions, (iii) Tra and Vir functions required for donor-recipient recognition and production of the structure through which the DNA-complex is transported. Due to detailed analyses of the RP4 conjugative machinery we provide a set of data confirming predicted analogies of both DNA Tra systems. It is important to note that the similarities include most of the processes directed from the donor cell such as cell to cell contact, initiation of DNA transmission, and DNA transport through membranes.

Our sequence comparison studies gave the first clue for an evolutionary and eventually functional relationship: TBS share a conserved region with the nick-region of *oriT*. The VirD operon is analogous to the relaxase operon including the first gene of the primase operon (*virD1/traJ, virD2/traI, virD3/traH* and *virD4/traG*). The VirC operon matches part of the leader operon (*virC1/traL*, and *virC2/traM*) and the VirB operon is similar to the Tra2 operon. Genes in these operons are arranged in the same order, although the relationship of the components can only be seen at the amino acid sequence level (10).

Evidence for functional analogies comes from a biochemical comparison of purified DNA processing components *in vitro*. Cleavage at *oriT*/TBS requires a superhelical substrate and the proteins TraJ/VirD1 and TraI/VirD2. Relaxases (TraI/VirD2) attach covalently to the DNA *via* tyrosines localized at the N-terminal moieties of the proteins. The relaxases are involved in initiation and termination of the transfer process. The reaction mode follows the same mechanistic principles (6, 11).

No such convincing experimental evidence is available for cell to cell contact and DNA transport functions. However, one promising aspect for an experimental approach comes from a mutational analysis of the *traG* gene. TraG has potential analogues in several conjugative systems including the TraD protein of the F complex and a Tra protein of a conjugative plasmid of a Gram-positive organism (12). The TraG-like proteins have motifs in common that resemble nucleotide binding folds. TraG point mutations in these motifs are transfer deficient for both RP4 and RSF1010 transfer indicating the functional and structural importance of these residues in a key process of DNA transfer.

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1 Cole, S. P., Lanka, E. & Guiney, D. G. (1993) *J. Bacteriol.* **175**:4911-4916.
2 Waters, V. L., Strack, B., Pansegrau, W. & Lanka, E. (1992) *J. Bacteriol.* **174**:6666-6673.
3 Lessl, M., Balzer, D., Lurz, R., Waters, V. L., Guiney, D. G. & Lanka, E. (1992) *J. Bacteriol.* **174**:2493-2500.
4 Lessl, M., Balzer, D., Weyrauch, K. & Lanka, E. (1993) *J. Bacteriol.* **175**:6415-6425.
5 Pansegrau, W., Balzer, D., Kruft, V., Lurz, R. & Lanka, E. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**:6555-6559.
6 Pansegrau, W., Schröder, W. & Lanka, E. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**:2925-2929.
7 Rees, C. E. D. & Wilkins, B. M. (1990) *Mol. Microbiol.* **4**:1199-1205.
8 Kotilainen, M. M., Grahn, A. M., Bamford, J. K. H. & Bamford, D. H. (1993) *J. Bacteriol.* **175**:3089-3095.
9 Stachel, S. E. & Zambryski, P. C. (1986) *Cell* **47**:155-157.
10 Lessl, M., Balzer, D., Pansegrau, W. & Lanka, E. (1992) *J. Biol. Chem.* **267**:20471-20480.
11 Pansegrau, W., Schoumacher, F., Hohn, B. & Lanka, E. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**:11538-11542.
12 Lessl, M., Pansegrau, W. & Lanka, E. (1992) *Nucleic Acids Res.* **20**:6099-6100.

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AGROBACTERIUM TUMEFACIENS TRANSFERS SINGLE-STRANDED T-DNA INTO THE PLANT CELL NUCLEUS

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In T-DNA transfer a defined piece of DNA, called T-DNA (transferred DNA), of the 200 kb Ti (tumor inducing) plasmid is moving from the bacterium to the plant. The T-DNA transfer is mediated by gene products of the virulence genes, located on the Ti plasmid outside the T-DNA. In *Agrobacterium* cells linear single- and double-stranded versions of the T-DNA have been detected that may be intermediates in the transfer process. Generally, preference is given to the single-stranded structure (T-strand) model, for several reasons: 1) strains missing the single-stranded DNA binding protein VirE2 can be complemented to virulence by coinoculation with a *vir*E2 containing strain (Otten et al., 1984) or by using a *vir*E2 transgenic plant as transformation recipient (Citovsky et al., 1992). The involvement of a single-stranded DNA binding protein in the transfer can be used as good argument for the single-strandedness of the transferred DNA. ii) Since single-stranded derivatives have been detected in induced agrobacterial cells, the transfer mechanism has been compared to conjugation of the bacterial F plasmid (Stachel and Zambryski, 1986; Albright et al., 1987; Zambryski, 1988). For the F conjugation system, a single-stranded molecules have never been detected in the donor cell (review; Willets and Skurray, 1987).

We recently showed that T-DNA is transferred as a single-stranded derivative from *Agrobacterium* to the plant cell nucleus. This conclusion is drawn from experiments exploiting the different properties of single- and double-stranded DNA (ss and dsDNA) to perform extrachromosomal homologous recombination in plant cells (Puchta and Hohn, 1991). After transfer from *Agrobacterium* to plant cells, T-DNA molecules recombined much more efficiently if the homologous sequences were of opposite polarity than if they were of the same polarity. This observation reflects the properties of ssDNA; ssDNA molecules of opposite polarity can anneal directly, whereas ssDNA molecules of the same polarity first have to become double stranded to allow annealing to take place. Judging form the relative amounts of single to double stranded T-DNA derivatives undergoing recombination, we infer that the D-DNA derivatives not only enter the plant nucleus in their single stranded form, but also that

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they most probably predominantly integrate as ssDNA molecules into the plant genome.

References

Albright, L.M., Yanofsky, M.F., Leroux, B., Ma, D. and Nester, E.W. (1987). Processing of the T-DNA of Agrobacterium tumefaciens generates border nicks and linear, single-stranded T-DNA. J. Bacteriol. **169**, 1046-1055

Citovsky, V., Zupan, J., Warnick, D. and Zambryski, P. (1992). Nuclear localization of Agrobacterium VirE2 protein in plant cells. Science **256**, 1802-1805

Otten, L., de Greeve, H, Leemans, J., Hain, R., Hooykaas, P. and Schell, J. (1984). Restoration of virulence of *vir* region mutants of Agrobacterium tumefaciens strain B6S3 by coinfection with normal and mutant Agrobacterium strains. Mol. Gen. Genet. **195**, 159-163

Puchta, H. and Hohn, B. (1991). The mechanism of extrachromosomal homologous recombination in plant cells. Mol. Gen. Genet. **230**, 1-7

Stachel, S.E. and Zambryski, P.C. (1986). Agrobacterium tumefaciens and the susceptible plant cell: A novel adaptation of extracellular recognition and DNA conjugation. Cell **47**, 155-157

Willetts, N. and Skurray, R. (1987). Structure and function of the F factor and mechanism of conjugation. In Escherichia cola and Salmonella typhimurium: cellular and molecular biology. F.C. Neidhart, J.L. Ingraham, K.B. Low, B. Magasanik, M. Schacchter and H.E. Umbarger, ed. (Washington, DC: American society for Microbiology) pp. 1110-1133

Zambryski, P. (1988). Basic processes underlying Agrobacterium-mediated DNA transfer to plant cells. Annu. Rev. Genet. **22**, 1-30

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II. Replication and Maintenance

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ROLLING CIRCLE-REPLICATING PROMISCUOUS PLASMIDS OF THE pMV158 FAMILY

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Many small plasmids replicate by an asymmetric rolling circle (RC) mechanism, characterized by replicative intermediates which have the newly synthesized leading strand covalently bound to the same parental strand (1-3). One example of these RC-replicons is the streptococcal plasmid pMV158 (4), and its Δ*mob* derivative, pLS1 (Figure 1), which have been autonomously established in more than ten different bacterial species. pMV158 is the prototype of a family of highly related RC-plasmids isolated (several of them recently) from gram-positive and -negative bacteria (Table 1). They share homologous motifs in their initiator of replication (Rep) proteins, and at one of the plasmid-control elements, the product of the *cop* gene. Sequence similarities are also found at their origins of replication. Although they do not exhibit homologous regions at their *inc* determinant (an antisense RNA), they have identical genetic organization at this region. Two plasmids of the this family, pLS1 and pFX2, show weak incompatibility in *Streptococcus pneumoniae*, and purified initiator RepB protein from pLS1 is able to relax supercoiled pFX2 DNA.

Table 1. The members of the pLS1 plasmid family

Plasmid	Host	Isolation	Size (bp)	Year
pMV158	Streptococcus agalactiae	USA	5536	1986
pWVO1	Lactococcus lactis	The Netherlands	2177	1991
pFX2	Lactococcus lactis	New Zealand	3536	1991
pE194	Staphylococcus aureus	Roumania	3728	1982
pLB4	Lactobacillus plantarum	UK	3548	1989
pA1	Lactobacillus plantarum	Yugoslavia	2820	1993
pLC2	Lactobacillus curvatus	Germany	2489	1993
pHPK255	Helicobacter pylori	UK	1455	1991
pADB201	Mycoplasma mycoides	Australia	1717	1989
pKMK1	Mycoplasma mycoides	USA	1875	1992

Replication of pMV158 is initiated by the plasmid-encoded RepB protein which introduces a strand- and site-specific nick at the unpaired region 5'-TACTACG/AC-3' (5,6), located on top of a secondary structure (Hairpin I) within the double-strand origin, *dso*. The nick leaves a free 3'-OH end which may be the substrate for host proteins to proceed with RC-replication. The end-products of this stage are a double-stranded plasmid molecule, and a ssDNA intermediate. These ssDNA plasmid forms are later converted to dsDNA molecules by the host machinery initiating at the single-strand

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origin (*sso*). In the case of pLS1, it seems that the pneumococcal RNA polymerase is involved in this conversion. The amount of ssDNA intermediates detected is plasmid- and host-dependent, and deletion of the *sso* leads to accumulation of ssDNA and to plasmid segregational instability (7,8).

Control of replication is exerted at two levels within the same circuit. At the translational level, the antisense RNA II, which is the main *inc* determinant, could interact with the *rep* mRNA initiation of translation signals. At the transcriptional level, the product of the *copG* (formerly *repA*) gene binds to and repress transcription from the *cop-rep* promoter, from which genes *copG* and *repB* are co-transcribed. However, plasmids defective in RNA II do not show any phenotypic defect, which points to a more complex mechanism (perhaps an intact CopG-RNA II circuit) to control the number of copies of pLS1 (9).

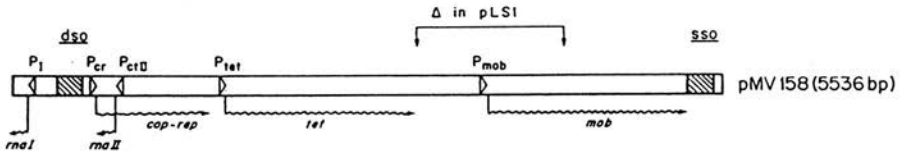

Figure 1. Genetic map of plasmid pMV158. Wavy lines, RNAs. Gene products translated from mRNAs: *cop-rep*, CopG and RepB; *tet*, TetL determinant (tetracycline-resistance); *mob*, Mob protein, involved in conjugative mobilization. The *maI*, *maII* antisense RNAs are also indicated. Other relevant features indicated are: dso, double-stranded origin; sso, single-stranded origin; open triangles, promoters. The region missing in pLS1 is indicated.

References.

1. Novick,R.P. (1989). Annu. Rev. Microbiol. **43**:537-565.
2. Gruss,A.D. and Ehrlich,S.D. (1989). Microbiol. Rev. **53**:231-241
3. del Solar,G., Moscoso,M. and Espinosa,M. (1993). Mol. Microbiol. **8**:789-796.
4. Lacks,S.A., López,P., Greenberg,B. and Espinosa,M. (1986). J. Mol. Biol. **192**: 753-765.
5. de la Campa,A.G., del Solar,G. and Espinosa,M. (1990). J. Mol. Biol. **213**:247-262.
6. M.Moscoso, G.del Solar and M.Espinosa. Accompanying Abstract.
7. del Solar,G., Puyet,A. and Espinosa,M. (1987). Nucl. Acids Res. **15**:5561-5580.
8. del Solar,G. Kramer,G., Ballester,S. and Espinosa (1993). Mol. Gen. Genet. **241**: 97-105.
9. G.del Solar and M.Espinosa. Accompanying Abstract.

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Maintenance systems of the Gram-positive broad-host-range plasmid pSM19035.

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The 27.2-kb low-copy-number plasmid pSM19035 and its derivative pDB101 (19-kb) have extraordinarily long inverted repeated sequences that comprise 80% and 76% of the molecule, respectively. The repeated sequences are separated by non-repeated sequences (NR_1 and NR_2) (Behnke et al., 1980). The antibiotic resistance marker is located in the relatively small NR_2 region. Both pSM19035 and pDB101 replicate by a theta mode (Ceglowski et al., 1993) and are stably inherited in Gram-positive bacteria with low G + C content. Removal of NR_1 and of one of the arms of the repeated region, which leads to the generation of pBT233, does not affect stable inheritance of the plasmid.

Analysis of the nucleotide sequence of pDB101 and its derivative pBT233 revealed thirteen major open reading frames (*orfs*). The *copS*, *repS*, *erm1* and *erm2* genes have been assigned to four of these *orfs*, giving the gene order *copS-repS-orfα-orfβ-orfγ-orfδ-orfε-orfζ-(erm2-erm1* or *erm1-erm2)-orfζ-orfε-orfδ-orfγ-orfβ-orfα-orfθ-orfη-orfι*. The organization of genes in the *orfθ-orfη-orfι-copS-repS-orfα-orfβ-orfγ* interval resembles the organization of genes in the *orfA* to *orfI* region of pAMβ1.

Plasmids pDB101, pIP501 and pAMβ1 belong to the same incompatibility group (*inc*18). Chimeric Rep proteins and replicons were obtained by domain swapping between *rep* genes and replicons of the above plasmid of the *inc*18 group (Brantl et al., 1990). The plasmid replication origin is located within a 45-bp segment immediately downstream of the initiation replication gene (Bruand et al., 1991; Ceglowski et al., 1993)

Deletion of a 1.5-kb segment of the *orfα-orfγ* interval does not seem to affect plasmid maintenance (segA region), but leads to the accumulation of plasmid multimers. Removal of a 3.0-kb fragment of the *orfδ-orfζ* region reduces plasmid segregational stability (segB region). Removal of both regions (segA and segB) abolishes it.

The maximization of random plasmid segregation is either accomplished by the recombination proficiency of the host or the presence of the pBT233 segA region. The segA region contains three protein coding frames (α, β and γ proteins). The β and γ proteins share homology with site-specific recombinases and type-I topoisomerases, respectively. We show that the β protein is able to mediate DNA resolution and DNA inversion in *E. coli*.

Better-than-random segregation requires an active segB region. The segB region contains three protein coding frames (δ, ε and ζ proteins). These *orfs* encode proteins of unknown activity, but of the δ protein certain domains share homology with domains observed in proteins associated with plasmid partitioning. These results suggest that pBT233 stabilization relies on a complex system involving resolution of plasmid oligomers (segA) and on the functions encoded by the segB region.

Northern blot analysis of the segA and segB regions led to the detection of mRNA species, the molecular weights of which correspond to *orfα* + *orfβ* and *orfγ* (segA) and *orf* δ and *orfε* + *orfζ* (segB) were detected. The putative promoters were fused to the *lacZ* gene. We show that the β protein exerts a negative effect on the utilization of the promoter

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region mapped upstream (*orf*α + *orf*β mRNA). *orf*γ is constitutively expressed from a weak promoter. Analysis of the segB region revealed that the synthesis of *orf*δ is subject to a negative control, and the synthesis of the *orf*ε + *orf*ζ mRNA species seems to be under a complex control mechanism.

A characterization of the seg A region revealed that the purified β protein binds co-operatively to its specific target sequence, which lies immediately downstream the plasmid replication origin. Protein β is a dimer in solution (Rojo et al., 1993). Between 3.6 to 4.2 β protomers are required to saturate the DNA substrate.

In *in vitro* analysis the purified β protein reveals the activity of a transcriptional repressor and, in the presence of a host factor, is able to catalyse intramolecular recombination between two specific sites on a supercoiled template. The β protein binding site, which is a 85-bp region, can be divided into two discrete subsites (sites I and II). Each subsite is about 34-bp in length, and they are separated from each other by 16-bp. Each of the subsites contains two 12 to 13-bp long imperfectly conserved sequences related to another by a dyad axis. When the β protein binds to its target site the DNA segment is bent or looped out. This indicates that the β protein interacts with itself within the protein-DNA complex.

Behnke et al. (1980) *Plasmid* 4,139, Brantl et al. (1990) *Nucleic Acids Res.* 18,4783; Bruand et al. (1991) *EMBO J.* 10,2171; Ceglowski et al. (1993) *FEMS Lett* 109,145; Rojo et al. (1993) *FEBS Lett.* 328,169.

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Role of Iteron Sequences in Initiation and Control of P1 Plasmid DNA Replication

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The hallmark for a class of bacterial plasmids is the presence of repeating sequences (iterons) in their origin of replication (reviewed in 1). Iterons serve as binding sites for the plasmid encoded initiator protein and, thereby, participate in the formation of higher order structures of iteron-initiator complexes, which is considered crucial for the initiation process (2,3). Iterons also control the frequency of initiation of plasmid replication in a cell generation and, thereby, maintain the plasmid copy number in a growing culture.

We have been interested to know whether iterons function only through interactions with the initiator or the sequences make diffusible products or recognize other factors. We have addressed these questions for the control function using the iterons of plasmid P1. Plasmid replication reduced drastically in the presence of a single 19 bp iteron, cloned in pUC19, and the results did not change when the iteron was flanked by transcription terminators. We conclude that a diffusible product from the iterons is not necessary for control. The question of accessory factor binding was addressed by generating a collection of mutant iterons (4). Mutations in any one of the highly conserved positions affected binding of the initiator, RepA, suggesting that the iteron sequences have been conserved primarily for this purpose (Fig). We also found that the binding and replication control activities of the mutant iterons are correlated. We infer that affinity for the initiator suffices to determine the control function. The idea of accessory roles of the sequences may still be correct and may require analysis of the initiation process.

The control activity of the iterons is believed to be mediated through pairing of iteron-initiator complexes (5,6). Pairing was evidenced in vitro by the demonstration of enhanced intermolecular ligation of iteron carrying fragments in the presence of RepA. The pairing reaction was most likely reversible since multimers were seen in the presence of ligase whether the fragments carried a single or multiple iterons. It appears that control by pairing is a simple reaction requiring only RepA and is driven primarily by iteron concentration, since RepA is autoregulated. This model, although adequate for correction of overreplication, does not explain how underreplication is avoided, which is specially important for the maintenance of a low copy number plasmid such as P1. Our attempts to answer this question will be discussed.

1. Kittel, B. L. & Helinski, D. R. (1993) in *Bacterial Conjugation*, ed. Clewell, D. B. (Plenum Press, New York), pp. 223-242.

Instituto Juan March (Madrid)

2. Mukherjee, S., Patel, I. & Bastia, D. (1985) *Cell* **43,** 189-197.
3. Mukhopadhyay, G. & Chattoraj, D. K. (1993) *J. Mol. Biol.* **231,** 19-28.
4. Papp, P. P., Chattoraj, D. K. & Schneider, T. D. (1993) *J. Mol. Biol.* **233,** 219-230.
5. Pal, S. K. & Chattoraj, D. K. (1988) *J. Bacteriol.* **170,** 3554-3560.
6. McEachern, M. J., Bott, M. A., Tooker, P. A. & Helinski, D. R. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 7942-7946.

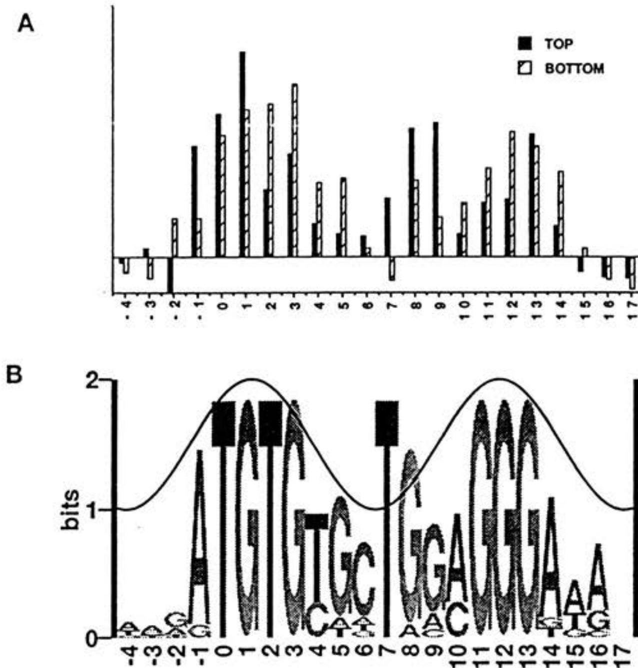

Fig. Comparison of the effect of base elimination on DNA binding by RepA (**A**) and the degree of base conservation among 14 natural iterons of P1 (**B**). (A) The bar heights represent relative degree of interference to RepA binding upon base elimination. (B) Bases are shown in a stack with heights in proportion to their relative frequencies (4). The height of each stack is the sequence conservation at that position in bits. A completely conserved base appears as a letter 2 bits high. The cosine wave represents one face of B-form DNA, the crests representing the major grooves. The correspondence between the bar heights (A) and conservation (B) is weak only at positions 7, 9, 15 and 16.

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REGULATION OF PARD, A KILLER STABILITY SYSTEM OF PLASMID R1

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parD is a killer stability system of the antibiotic resistance factor R1 that is close to the basic replicon of the plasmid (1). This system, that is related to the *ccd* stability system of plasmid F (2), contains two genes, *kis* and *kid*, that code for proteins of 9.3 kDa and 12 kDa respectivelly. Kid is a killer determinant and Kis a supressor of the killing mediated by Kid (3). Transcription of *parD* initiates from a single promoter and originates two *parD* transcripts: a polycistronic transcript of 700 b and a shorter transcript of 370 b coding only for the first component of the system. These *parD* transcripts are present in similar amounts and have short and similar half lifes; this results in double transcriptional dosage for the *parD* antagonist than for the *parD* killer (4). The 3´ end of the short *kis* transcript (370 b), is located after a stem-loop sequence situated close to the 5´ end of the *kid* gene. *In vitro* and *in vivo* analysis indicate that this short *kis* transcript is mainly formed by degradation of the polycistronic *parD* transcript and not by transcriptional termination promoted by the 3´ steam-loop structure. The following data indicate that synthesis of the killer protein is translationally coupled to synthesis of the *parD* antagonist: an ambar mutation that truncates synthesis of the Kis protein at an early stage, or a mutation than changes the termination codon of the *kis* gene and results in overlapping of the *kis* and *kid* genes, lead to > 90% reduction in the levels of Kid protein. The ambar mutation in *kis* reduces slightly transtription of the *kid* gene indicating that transcriptional polarity regulate also expression of the *parD* genes. It can be concluded that post-transcriptional mechanisms modulate the differential expression of the two *parD* genes favouring greater levels of the antagonist than of the killer protein (Ruiz-Echevarría, unpublished results).

Transcription of the *parD* operon is repressed by the coordinate action of the Kis and Kid proteins (4). The target of this autoregulatory loop are inverted repetitions present in the *parD* promoter region. This target has been defined *in vitro* by gel retardation and DNA-footprinting analysis using *in vitro* synthesized wild-type *parD* proteins and a *parD* promoter fragment of 350 bp (De la Cueva, unpublished results).

The wild-type *parD* system is transcriptionally repressed, and inactive, under normal replication conditions, but this system can be transcriptionally derepressed by a mutation in *repA* that reduces the efficiency of plasmid replication. This derepression, that can be reversed

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by a copy-up mutation (*copB* mutant), is associated with and increase in plasmid stability (Ruiz-Echevarría *et al*, submitted). These results indicate that the *parD* wild-type system can shift from a repressed (inactive) to a derepressed (active) situation as results of mutations that interfere with plasmid replication. The importance of *parD* derepression for the active role of *parD* in plasmid stabilization was previously suggested by the analysis of a derepressed *parD* mutant (1).

(1) Bravo,A., de Torrontegui,G., and Díaz,R. Identification of components of a new stability system, *parD*, of plasmid R1 that is close to its origin of replication. Mol. Gen. Genetics 210, 101-110 (1987)
(2) Ruiz-Echevarría,M.J., de Torrontegui,G., Giménez-Gallego,G., and Díaz-Orejas,R. Structural and functional comparison between the stability systems *parD* of plasmid R1 and *ccd* of plasmid F. Mol. Gen. Genetics, 225, 355-362 (1991)
(3) Bravo,A., Ortega,S., de Torrontegui,G., and Díaz,R. Killing of *Escherichia coli* cells modulated by components of the stability system *parD* of plasmid R1. Mol. Gen. Genetics 215, 146-151 (1988)
(4). Ruiz-Echevarría, M.J., Berzal-Herranz,A., Gerdes,K. and Díaz-Orejas,R. The *kis* and *kid* genes of *parD* maintenance system of plasmid R1 form an operon that is autoregulated at the level of transcription by the coordinate action of the Kis and Kid proteins. Mol. Microbiol. 5, 2685-2693 (1991)

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The ccd killer system of plasmid F.

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DNA topoisomerases perform essential roles in DNA replication, gene transcription and chromosome segregation. They are the targets of potent molecular therapeutic agents, including the quinolone antibiotics that target gyrases and antitumor agents that target mammalian topoisomerases I and II. Recently, we identified a new type of topoisomerase II poison: the CcdB protein of plasmid F. The *ccd* locus contains two genes ccdA and ccdB whose gene products are involved in a poison-antipoison mechanism. When its action is not prevented by CcdA protein, the CcdB protein is a potent cytotoxin. Using purified CcdA, CcdB and gyrase, we show that CcdB efficiently traps gyrase in a cleavable complex. The CcdA protein not only prevents the gyrase poisoning activity of CcdB but also reverses its effect on gyrase. The mechanism by which the CcdB protein induces gyrase-mediated DNA strand breakage is closely related to the action of quinolone antibiotics. However, the ATP dependence of the CcdB cleavage process differentiates the CcdB mechanism from quinolone-dependent reactions. ATP is required at its hydrolysis site on the GyrB subunit, showing that GyrB subunit plays a role in CcdB-dependent cleavage. However, GyrA protein is primarily responsible for determinig the sensitivity of gyrase to the CcdA/CcdB plasmid killer system. The strongest evidence that the GyrA subunit interacts with CcdB is that gyrase reconstituted with the mutant GyrA462 subunit, selected for a CcdB resistant phenotype *in vivo*, was refractory to the CcdB-induced gyrase DNA cleavage *in vitro*.
The *ccd* locus contributes to the stability of plasmid F by postsegregational killing of plasmid-free bacteria. Using pulse-chase experiments, we have obtained direct evidence that the half-life of CcdA is shorter than that of CcdB. Using the same technique with protease mutant strains ($HflA^-$, $ClpP^-$ and Lon^-), we have shown that CcdA is stable in a Lon^- strain. This result strongly suggest that Lon is the major protease responsible for degradation of CcdA. Moreover, our results indicate that this specific proteolysis of CcdA is required for *ccd*-mediated postsegregational killing and SOS induction. This allows the conclusion that Lon-dependent proteolysis of CcdA is the key control for activation of CcdB in plasmid-free segregant bacteria.
The *ccdA* and *ccdB* genes are organized in an operon and their expression is negatively autoregulated at transcription level (Tam and Kline, 1989; de feyter et al., 1989). In order to define functional domains of the CcdA and CcdB proteins involved in autoregulation, poison-antipoison activities and susceptibilty to Lon protease, we have isolated and sequenced mutants affected in these properties. Results of this analysis will be presented in the poster.
A collection of 260 strains containing plasmids representative of the following Inc groups: IncB, IncC, IncD, IncFI, IncFII, IncFIII, IncFIV, IncFme, IncFV, IncFVI, IncFVII, IncHI1, IncHI2, IncHII, IncI1, IncI2, IncIg, IncJ, IncK, IncM, IncN, IncP, IncQ, IncSI, IncSII, IncSIII, IncSIV, IncT, IncU, IncV, IncW, IncX, IncY and IncZ was used to screen for the presence of the *ccd* locus by DNA-DNA hybridization experiment. The *ccd* locus hybridized only with the plasmids carrying a RepFIA type replicon except the IncHI1 plasmids.

Recent publications:

-J. Steyart, L. Van Melderen, P. Bernard, M.H. Dao THI, R. Loris, L. Wyns and M. Couturier. J. Mol. Biol. 1993. **231**: 513-515.
-P. Bernard, K. Kézdy, L. Van Melderen, J. Steyart, L. Wyns, M.L. Pato, P.N. Higgins and M. Couturier. J. Mol. Biol. 1993. **234**: 534-541.
-L. Van Melderen, P. Bernard and M. Couturier. Mol. Microbiol., in press.
-M. Salmon, L. Van Melderen, P. Bernard and M. Couturier. Mol. Gen. Genet., submitted.

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Antisense RNA Regulated Programmed Cell Death.

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The *hok/sok*, *srnB* and *pnd* systems of plasmids R1, F and R438 mediate plasmid maintenance by killing of plasmid-free segregants. The systems encode exceptionally stable mRNAs which can be translated into cell toxins (Hok, SrnB', and PndA) that kill the cells from within. Translation of the killer mRNAs is regulated by small unstable antisense RNAs that are complementary to the leader-regions of the killer mRNAs. The differential decay rates of the mRNAs encoding the toxins and the inhibitory antisense RNAs explain the killing of plasmid-free segregants and the induction of these systems by the addition of rifampicin. The antisense RNAs (Sok, SrnC and PndB) inhibit translation of the toxin encoding mRNAs indirectly by inhibiting translation of reading frames that overlap with the toxin-encoding genes (*mok*, *srnB*, and *pndC*; see Figure 1). The initial recognition reaction between Sok-RNA and Hok mRNA takes place between the single-stranded 5'-end of Sok-RNA and the complementary region in Hok mRNA (the *mok* TIR), without the involvement of an antisense RNA loop in the initial binding step (Thisted et al., 1994a).

In addition to the full-length mRNAs, the killer systems also produce truncated mRNAs that are shortened by 35 - 70 nucleotides in the 3'-ends. The appearance of the truncated mRNAs is correlated with expression of the toxins *in vivo*. Figure 1 shows the proposed local secondary structures of the full-length and truncated mRNAs. Using *in vitro* synthesized mRNAs we have shown, that the truncation occurs by (slow) processing in the 3'-ends of the mRNAs. Most importantly, the full-length mRNAs are translationally inactive *in vitro*, while the truncated mRNAs are translated efficiently (Thisted et al., 1994b). Secondary structure analyses of Hok mRNA suggest that the 3'-end of the full-length mRNA encodes an anti-Shine & Dalgarno sequence that folds back onto the *mok* TIR and represses translation of *mok* (and thereby *hok*). By analogy, we suggest that the *pnd* and *srnB* mRNAs are regulated in a similar way.

Why are the killer gene systems equipped with this extra control loop superimposed on the regulation by antisense RNAs? Normally, antisense RNAs inhibit their target RNAs by forming stable duplexes with the target molecules. Furthermore, rapid cleavage of the antisense RNA:target RNA duplexes by RNase III adds to the irreversible nature of this type of inhibition (see e.g Blomberg et al., 1990). Obviously, translation of *hok* is activated after decay of Sok-RNA (in plasmid-free cells and after addition of rifampicin). The key to this activation seems to be the presence, in steady state, of a pool of full-length, translationally inactive Hok mRNA that has not yet reacted with Sok-RNA. Therefore, we suggest that the fold-back structure in the full-length Hok mRNA has dual functions: On the one hand, the long-range fold-back interaction with the *mok* TIR prevents translation of *mok* which otherwise would result in cell killing, and on the other hand it prevents Sok-RNA from binding to the same TIR region. Rapid binding of Sok to the full-length mRNAs would be detrimental for functioning of the system, since the Sok:Hok mRNA duplex is rapidly cleaved by RNase III (Gerdes et al., 1992). Thus, when plasmid-free cells appear (or after addition of rifampicin) the inhibitory Sok-RNA decays rapidly, leaving behind the pool of inactive full-length Hok mRNA. These molecules are then slowly processed in their 3'-ends, thereby leading to accumulation of the translationally active, truncated Hok mRNA. Hence, production of toxin and cell killing ensues. The programmed cell death mediated by the plasmid encoded killer gene systems is a simple differentiation process which is now understood at the molecular level. The model outlined above is depicted in Figure 2.

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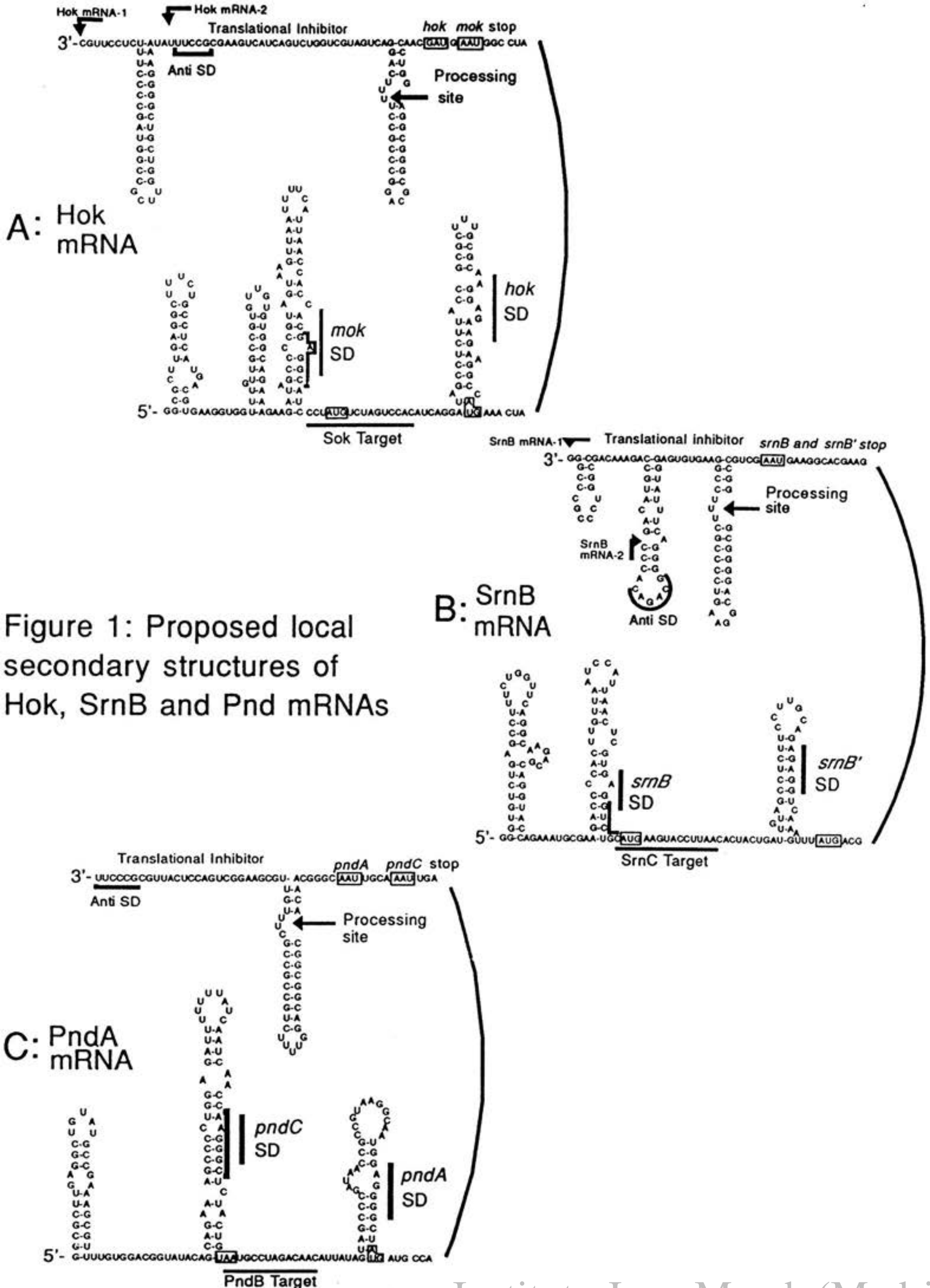

Figure 1: Proposed local secondary structures of Hok, SrnB and Pnd mRNAs

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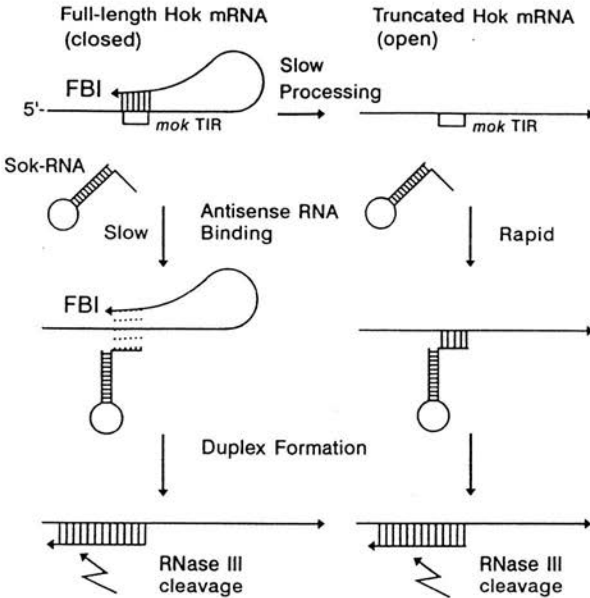
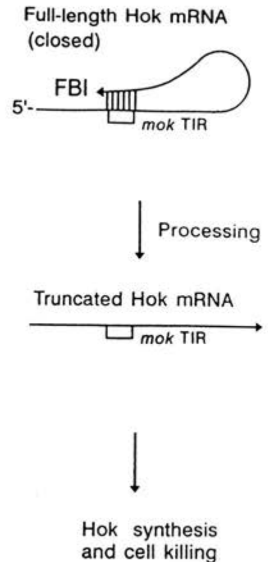
FIGURE 2: MODEL THAT EXPLAINS ACTIVATION OF hok TRANSLATION IN PLASMID-FREE CELLS.





References:

Gerdes, K., Nielsen, A., Thorsted, P., and Wagner, E.G.H. (1992). Mechanism of killer gene activation: Antisense RNA-dependent RNase III cleavage ensures rapid turn-over of the stable Hok, SrnB and PndA effector mRNAs. J. Mol. Biol. 226, 637-649.

Thisted, T., Sørensen, N., Wagner, E.G.H., and Gerdes, K. (1994a). Mechanism of postsegregational killing: Sok antisense RNA interacts with Hok mRNA *via* its 5'-end single-stranded leader and competes with the 3'-end of Hok mRNA for binding to the *mok* translation initiation region. Submitted to EMBO J.

Thisted, T., Nielsen, A., and Gerdes, K. (1994b). Mechanism of postsegregational killing: Translation of Hok, SrnB, and PndA mRNAs of plasmids R1, F, and R483 is activated by 3'-end processing. Submitted to EMBO J.

Blomberg, P., Wagner, E.G.H., and Nordström, K. (1990). Control of replication of plasmid R1: the duplex between the antisense RNA, CopA, and its target, CopT, is processed specifically in vivo and in vitro by RNase III. EMBO J. 9, 2331-2340.

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Molecular Genetics of Broad Host Range IncP Plasmids

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Many IncP plasmids can transfer themselves efficiently and maintain themselves very stably in a diverse range of Gram negative bacterial species. (1). This family of plasmids contains two main branches termed IncPα and InPβ. The best studied IncPα plasmids are the indistinguishable 60kb plasmids RP1, RP4, RK2, R18 and R68 which all originated in Birmingham and confer resistance to penicillin, tetracycline and kanamycin. The best characterized IncPβ plasmid is R751 (53kb) which confers trimethoprim resistance. Comparison of these IncPα and IncPβ plasmids allows the backbone of essential plasmid function to be identified. This backbone consists of genes for replication and transfer whose expression is coordinated by a central control operon which also appears to encode an active partitioning apparatus.

Replication functions: Replication of IncP plasmids depends on two loci: *oriV*, the *cis*-acting origin of replication; and *trfA*, which encodes proteins necessary to activate *oriV*. Comparison of IncPα and IncPβ plasmids shows that in the ancestral IncP plasmid these two loci were clustered, a transcriptional terminator at the end of *trfA* protecting *oriV* from transcription originating at the strong *trfA* promoter which can inhibit initiation of replication (2). The replication origin consists of a series of five iterons, to which TrfA protein binds, an AT- and a GC-rich region and three DnaA binding sites (3,4). In *Pseudomonas aeruginosa* the pair of DnaA binding sites upstream of the TrfA iterons is not required but it is essential in all other species tested (5,6). *trfA* contains two translational starts (7,8). The shorter product is sufficient for replication in most species but the larger product is necessary for efficient maintenance in *P. aeruginosa* (9,10). Only the smaller product is highly conserved between IncPα and IncPβ plasmids, although the feature of two translational starts is present in IncPβ plasmids as well.

Partitioning functions: In IncPα plasmids the primary segregational stability functions are encoded by the *par*/*mrs* region which appears to include a site specific recombinase to resolve multimers, an active partitioning apparatus and a pair of genes which may result in killing of plasmid-free segregant bacteria (11,12,13). However this *par*/*mrs* region is not conserved in IncPβ plasmids which nevertheless is stably maintained. An alternative partitioning system is provided by genes in the central control operon region, which will stabilize heterologus, low copy number plasmids (14). The IncC protein encoded in this region is related to the SopA and ParA proteins of F and P1 respectively, which belong to a family of ATPases involved in partitioning and cell division. KorB, the second component of this *par* system, shows weak similarity to the SopB and ParB proteins of F and P1 respectively which are DNA binding proteins that interact specifically with the *cis*-acting centromere-like sequences in these plasmids. In RK2 three other proteins are implicated: KfrA, a site specific DNA binding protein with an extended α helical coiled-coil C-terminal domain (15); and KorFI and KorFII, which are histone-like proteins (16). KorB, KfrA, KorFI

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and KorFII all bind near the *kfrA* promoter region at the end of the *KorABF* operon and this may be the *cis*-acting site needed for partitioning.

Control: Proteins KorA and KorB, encoded in the central control operon, play a key role in coordinating IncP plasmid gene expression. The *korABF* operon is autoregulated, providing a means of maintaining a balance of these proteins. KorB binds at 12 sites around the plasmid genome (17, 18). Where these sites occur near promoters KorB represses transcription and thus coordinates expression of a number of operons including the *korABF* operon (control/partitioning), the *trfA* operon (vegetative replication), the *trb* operon (conjugative transfer) and the *krfA* operon (partitioning?). KorA binds to an operator which occurs 7 times on the genome. All these sites are in promoter regions and it also represses transcription of the *korABF*, *trfA* and *kfrA* operons. However, when it represses *trfAp* it stimulates *trbAp*, a promoter for the *trb* operon which is organized as a face to face divergent promoter with the *trfAp* (19). The first gene of the *trb* operon is *trbA* which encodes a DNA binding protein that represses transcription from *trbB*, the second promoter in the *trb* operon as well *trfAp* (20), and *traKp*, *traJp* and *traGp*, the *tra* operon promoters (21). This complex network of interactions serves to coordinate and balance all the basic transfer and maintenance genes of the plasmid. Our observations that different regulators respond differently to changing physiological conditions suggest that these circuits may also provide a response to external factors which could favour one type of propagation (replication or transfer) over the other.

References

1. Thomas, C.M. and Smith, C.A. (1987) Ann. Rev. Microbiol. 41, 77-101.
2. Smith, C.A., Pinkney, M. and Thomas, C.M. (1993) J. Gen. Microbiol. 139, 1761-1766.
3. Stalker, D.M., Thomas, C.M. and Helinski, D.R. (1981) Mol. Gen. Genet. 181, 8-12.
4. Gaylo, P.J., Turjman, N. and Bastia, D. (1987) J. Bacteriol. 169, 4703-4709.
5. Cross, M.A., Warne, S.R. and Thomas, C.M. (1986) Plasmid 15, 132-146.
6. Nash, J. and Krishnapillai, V. (1987) Plasmid 18, 35-45.
7. Shingler, V. and Thomas, C.M. (1984) J. Mol. Biol. 175, 229-250.
8. Smith, C.A. and Thomas, C.M. (1984) J. Mol. Biol. 175, 251-262.
9. Durland, R. and Helinksi, D.R. (1987) Plasmid 18, 164-169.
10. Shingler, V. and Thomas, C.M. (1989) Biochim. Biophys. Acta. 1007, 301-308.
11. Gerlitz, M., Hrabak, I. and Schwab, H. (1990) J. Bacteriol. 172, 6194-6203.
12. Roberts, R.C., Burioni, R. and Helinski, D.R. (1990) J. Bacteriol. 172, 6204-6216.
13. Roberts, R.C. and Helinski, D.R. (1992) J. Bacteriol. 174, 8119-8132.
14. Motallebi-Veshareh, M., Rouch, D.A. and Thomas, C.M. (1990) Mol. Microbiol. 4, 1455-1463.
15. Jagura-Burdzy, G. and Thomas, C.M. (1992) J. Mol. Biol. 225, 651-660.
16. Jagura-Burdzy, G., Ibbotson, J.P. and Thomas, C.M. (1991) J. Bacteriol. 173, 826-833.
17. Balzer, D., Ziegelin, G., Pansegrau, W., Kruft, V. and Lanka, E. (1992) Nucl. Acids Res. 20, 1851-1858.
18. Williams, D.R., Motallebi-Veshareh, M. and Thomas, C.M. (1993) Nucl. Acids Res. 21, 1141-1148.
19. Jagura-Burdzy, G. and Thomas, C.M. (1994) Submitted.
20. Jagura-Burdzy, G., Khanim, F., Smith, C.A. and Thomas, C.M. (1992) Nucl. Acids Res. 20, 3939-3944.
21. Zatyka, M., Jagura-Burdzy, G. and Thomas, C.M. In preparation.

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III. Plasmid Transfer

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Coordination of promiscuous plasmid replication and transfer: regulatory protein KorA of broad host range plasmid RK2 acts both positively and negatively at the divergent *trfA/trbA* promoter region

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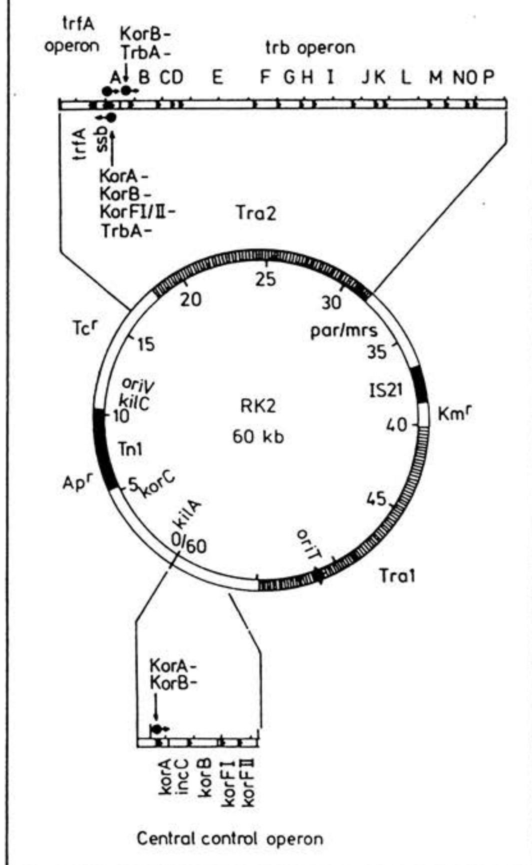
IncP plasmids are capable of transfer between and maintenance in almost all Gram negative bacterial species (1). IncP plasmids are found in many natural environments and thus could be responsible for promiscuous spread of rDNA during deliberate release experiments. The expression of at least some of the IncP plasmid transfer genes is coordinated with those for vegetative replication (2,3) and therefore provide an interesting system to study factors influencing the balance of plasmid spread and maintenance.

The particular IncP plasmid we have been studying is RK2, which is virtually identical to RP1, RP4, R18 and R68, the best characterized IncPα plasmids (1). The specific genes referred to above are the *trfA* operon and the *trb* operon located next to each other on the RK2 genome (Figure 1). The *trfA* operon consists of *ssb* (or *san*) encoding a single stranded DNA binding protein, and *trfA*, whose polypeptide products are essential to activate the replication origin, *oriV* (4,5). We have shown that *trfAp* is repressed by KorA , KorB, KorFI and KorFII, which are all encoded in the central control operon (6,7). The *trb* operon consists of 15 cistrons which are primarily involved in mating pair formation during conjugative transfer (8,9). There are two promoters near the start of the operon, preceding *trbA* and *trbB* respectively. The *trbB* promoter has been shown to be repressed by KorB (2). *trbA* encodes an additional regulatory protein which appears to modulate both *trfA* and *trbB* expression (3). Transcription from *trbAp* is likely to continue into *trbB* and beyond (6), and will

Figure 1. Map of RK2 showing the organization of the *trfA* operon , the *trb* operon and the central control operon.



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generate mRNA containing secondary structure which is absent from the *trbBp*-generated message (2). *trbAp* activity is therefore likely to be of crucial importance in the balance of activity of these operons.

The poster will describe dissection of the *trfAp/trbAp* region. The *trfAp* and *trbAp* are arranged as face to face divergent promoters. Deletion mapping and mutation analysis established the location of *trbAp*. Primer extension mapping of *tsp* and permanganate footprinting confirmed the results of *in vivo* studies. The *korA* gene, which is known to repress seven operons on RK2, including the *trfA* operon, was shown to stimulate *trbAp*. The effect of *korA* on *trbAp* is mimicked by the *trfAp-1* promoter down mutation, suggesting that a reduction in the strength of *trfAp* is required for derepression of *trbAp* activity. The inhibitory effect of strong *trfAp* on *trbAp* is neither due to RNAP exclusion nor inhibition of open complex formation at *trbAp* since the *trfAp-1* mutation, which reduces RNAP binding and open complex formation at *trfAp* but does not stimulate melting at *trbAp in vitro*. KorA has been purified and shown by gel retardation and DNaseI footprinting to bind a single operator region containing the sequence 5'TTTAGCTAAA3', which overlaps the -10 hexamer of the *trfA* promoter. Binding of KorA at this operator does not exclude RNA polymerase from *trfAp*. Footprinting also suggests that KorA introduces the bend in DNA between two promoters. The results described in this poster lead us to conclude that KorA must affect the balance between *trfA* and *trb* operon transcription in a completely different way from the previously observed effects of KorB and TrbA which repress both operons.

A major long term goal of this work is to explain and predict plasmid behaviour (copy number, stability and transfer frequency) under different physiological conditions and in a range of species. Before quantitative studies on levels of transcripts and regulatory proteins are carried out to correlate control of gene expression to plasmid functions the molecular components of the circuits need to be defined. We believe that the identification of this KorA-mediated genetic switch described here is a key step in this process.

REFERENCES

1. Thomas, C.M. & Smith, C.A. (1987) Ann. Rev. Microbiol. 41, 77-101.
2. Motallebi-Veshareh, M., Balzer, D., Lanka, E., Jagura-Burdzy, G. & Thomas, C.M. (1992) Mol. Microbiol.
3. Jagura-Burdzy, G., Khanim, F., Smith, C.A. & Thomas, C.M. (1992) Nucl. Acids Res. 6, 906-920.
4. Shingler, V. & Thomas, C.M. (1984) J. Mol. Biol. 175, 229-250.
5. Smith, C.A. & Thomas, C.M. (1984) J. Mol. Biol. 175, 251-262.
6. Shingler, V. and Thomas, C.M. (1984) Molec. Gen. Genet., 195, 523-529.
7. Jagura-Burdzy, G., Ibbotson, J.P. and Thomas, C.M. (1991) J.Bacteriol., 173, 826-833.
8. Lessl, M., Balzer, D., Lurz, R., Waters, V., Guiney, D.G. and Lanka, E. (1992) J.Bacteriol. 174, 2493-2500
9. Lessl, M., Balzer, D., Pansegrau, W. & Lanka, E. (1992) J. Biol. Chem. 267, 20471-20480.

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Mechanisms allowing plasmids to evade restriction barriers to transfer

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Restriction systems operate in bacteria to destroy infecting foreign DNA, identified as alien by its lack of modification methylation at enzyme specificity sites. Since approximately one quarter of all bacterial isolates have been shown to encode a restriction system and almost 200 different types of specificity site have been identified (Wilson & Murray, 1991), restriction must constitute a frequently encountered barrier to DNA transfer in the wild. Plasmids transferred by bacterial conjugation are subject to restriction but can evade the process by at least three different mechanisms.

Elimination of restriction sites is one evasion strategy. This may result from selection, operating each time an unmodified plasmid transfers to a restricting host. Selection against restriction sites has been demonstrated in bacteriophage infections (Murray & Murray, 1974) and proposed to occur in phage evolution (Sharp, 1986). Broad host-range plasmids of the IncP group are remarkably deficient in sites recognised by many different type II restriction enzymes, suggesting that extensive contraselection of sites has occurred through a history of intergeneric conjugations (Meyer *et al.*, 1977). A contrasting explanation is suggested by our computer-aided analysis of nucleotide sequence motifs in the Tra1 and Tra2 regions of RP4. Sequence data were provided by Erich Lanka's laboratory. The results show that the RP4 transfer region is deficient of many palindromic sequences typical of type II restriction targets but other short symmetrical sequences are overabundant. A similar pattern of deficiencies and abundancies is present in chromosomal DNA of *Pseudomonas aeruginosa*, a natural host of IncP plasmids. RP4 Tra is further depleted of certain hexanucleotide palindromic sequences, a number being targets of known restriction enzymes of *P. aeruginosa*. These findings suggest that biased frequencies of sequence motifs in a plasmid are caused in part by mutational pressures operating on DNA within a preferred host type and, further, by selection operating during historic transfers between closely related bacteria. It is unnecessary to invoke promiscuous transfers between different genera as the sole explanation.

Plasmids can also evade restriction by a process dependent on conjugative transfer of more than one copy of the plasmid. The process is non-specific in acting against at least type I and type II restriction systems and presumably involves some breakdown of the restriction mechanism in response to an early event in conjugation. Transfer of enzyme-saturating amounts of DNA might be the responsible factor (Read *et al.*, 1992). Other physiological explanations should be considered, since expression of restriction (R) but not modification (M) activity is delayed extensively in transconjugant cells following acquisition of type I R-M genes (Prakash-Cheng & Ryu, 1993).

Carriage of an anti-restriction gene on the plasmid constitutes a third type of restriction-evasion mechanism. One such gene is located on the IncI1 plasmid ColIb-P9 (Delver *et al.*, 1991; Wilkins *et al.*, 1991). The locus, called *ardA*, specifically alleviates restriction of DNA by all three families of type I restriction

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enzyme. Genetic evidence shows that carriage of *ard* allows unmodified ColIb to evade restriction following its conjugative transfer to recipients specifying the type I enzyme *Eco*K. The gene maps in the ColIb leading region, defined as the first segment of the plasmid to enter the recipient cell during conjugation. This is the expected location of a gene that is expressed early in the transconjugant cell to promote installation of the immigrant plasmid (Read *et al.*, 1992).

We have examined the distribution of *ardA* on representatives of 23 different incompatibility groups of conjugative plasmid. The test was for hybridisation to a probe of a PCR-amplified sequence of ColIb *ardA* determining a 22 kDa polypeptide in a coupled *in vitro* transcription-translation system. Hybridising sequences were found on members of the IncI1-B-K subset of the I complex of plasmids, on an IncFV plasmid of the F complex, and on IncN group plasmids. The *ardA*-hybridising sequence on the B and on the FV plasmid are ORFs with at least 63% sequence identity to the ColIb gene. ORF-containing fragments of the B and FV plasmids map in the leading region and confer an anti-restriction phenotype on vegetative cells. The same properties have been reported for the *ardA* gene on an IncN plasmid (Belogurov *et al.*, 1992). Why should *ard* homologues be present on plasmids of the I complex, F complex and the IncN group, which are considered to represent different evolutionary lineages? These plasmids, together with members of the IncX group, are authentic plasmids of *Escherichia coli* and close relatives, as judged by the plasmids present in the Murray collection of bacteria isolated in the 'pre-antibiotic' era (Datta & Hughes, 1983). A similar cluster of enterobacteria is the only known source of type I restriction enzymes (Wilson & Murray, 1991). Hence, ArdA appears to be an adaptation allowing conjugative plasmids to escape a type of restriction system determined by a particular cluster of host bacteria, rather than a general mechanism for restriction evasion.

References:

Belogurov, A.A., Delver, E.P. & Rodzevich, O.V. (1992) *J. Bacteriol.* 174:5079-5085.
Datta, N. & Hughes, V.M. (1983) *Nature* 306:616-617.
Delver, E.P., Kotova, V.U., Zavilgelsky, G.B. & Belogurov, A.A. (1991) *J.Bacteriol.* 173:5887-5892.
Meyer, R., Figurski, D. & Helinski, D.R. (1977) *Molec. Gen. Genet.* 152:129-135.
Murray, N.E & Murray, K. (1974) *Nature* 251:476-481.
Prakash-Cheng, A. & Ryu, J. (1993). *J. Bacteriol.* 175:4905-4906.
Read, T.D., Thomas, A.T. & Wilkins, B.M. (1992) *Mol. Microbiol.* 6:1933-1941.
Sharp, P.M. (1986) *Mol. Biol. Evol.* 3:75-83.
Wilkins, B.M., Rees, C.E., Thomas, A.T. & Read, T.D. (1991) *Plasmid* 25:227.
Wilson, G.G. & Murray, N.E. (1991) *Annu. Rev. Genet.* 25:585-627.

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Environmental factors affecting plasmid and gene transfer in filamentous soil bacteria

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The streptomycetes are a group of soil bacteria well adapted to survival in soil as they produce extensive networks of hyphae able to colonise and derive nutrients over a large surface area. In addition they produce spores which are resistant to phage infection and various toxins such as antibiotics. This life cycle has advantages in environments where nutrients are scarce and discontinuous in time and space. Studies of plasmid and phage ecology have shown that these conditions favour lysogeny and plasmid transfer. However plasmid transfer frequencies were difficult to measure acurately due to the plasmid spread function within the mycelium. Under conditions of selection for plasmid or phage-borne genes gene transfer frequencies were not increased but there was evidence for changes in copy number. All studies were done with antibiotic resistance genes where selection was achieved by additions of antibiotics to soil. Streptomycetes can produce a wide range of antibiotics and frequently show resistance to several antibiotics. Studies of the distribution of these genes in natural populations has indicated retrospective eidence for transfer of production and resistance genes. The mechanism of transfer is uncertain but may involve giant linear plasmids.

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GENETIC TRANSFER IN WATER

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Up to about 70% of aquatic bacteria contain plasmids and many of these are large and so possible conjugative. So it is likely that these extra-chromosomal elements play an important role in populations of water bacteria. Early experiments designed to study conjugal gene transfer in water relied mainly upon plasmids commonly used by geneticists, which were often obtained from clinical sources. More recently plasmids isolated directly from aquatic communities have been used. There are two major ways of isolating plasmids from indigenous aquatic bacteria (Fry & Day, 1990). Endogenous isolation depends on screening isolates from plating or enrichment experiments for the physical presence of large plasmids. These are then tested for transfer ability with standard laboratory mating procedures. In exogenous isolation a natural community and a suitable plasmid free recipient are incubated together. During this incubation conjugative plasmids are transferred to the recipient. Transconjugants can then be obtained by plating on a medium with selective agents for both the recipient and putative plasmid phenotype. A mixture of rifampicin for the recipient and Hg for the plasmid works well in many cases. Exogenous isolation has proved an invaluable procedure as it consistently provides large, conjugative plasmids which transfer at high frequency.

Many attempts have been made to classify natural plasmids obtained by the methods outlined above. However, at present no method has proved very satisfactory. About two-thirds of exogenously isolated plasmids seem to be broad host range and only one third have narrow host ranges. However, unlike RP4 which transfers into almost all Gram -ve negative recipients, natural aquatic plasmids transfer into a narrower range of widely differing Gram negative hosts. Restriction digest profiles have consistently revealed groups of structurally similar plasmids. However, hybridization for the classical *Escherichia coli* incompatibility groups has not proved useful with plasmids isolated in more typical, naturally occurring aquatic genera like *Psuedomonas,* as very few plasmids isolated probed positively. Very recent studies using probes made from whole plasmids and restriction fragments of plasmids appear to present a more promising approach to classifying natural aquatic plasmids.

Studies of plasmid transfer in water have usually involved counting transconjugants after incubation of a donor and recipient bacterium together. These experiments have slowly become more realistic. They started with mating pairs incubated *in situ* in enclosed mating chambers without indigenous bacteria and have progressed to more natural experiments with the mating pairs exposed to the full range of biotic and abiotic factors that the environment presents. Experiments of this type with conjugative plasmids have given transfer frequencies as high as 2×10^{-1} transconjugants per recipient. Many factors have been found to effect this frequency, but temperature and donor to recipient ratio seem to have the greatest affect (Bale *et al.*, 1988; Fernandez-Astorga *et al*, 1992). In temperate rivers temperature seems much more important than factors such as pH and nutrient availability. Realistic microcosm experiments have also been designed which have largely confirmed these findings and have also proved useful in studies using recombinant organisms or plasmids which cannot easily be

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used *in situ*. These microcosm experiments have also shown that conjugal plasmid transfer occurs predominantly in submerged surface biofilms, and not in the freely suspended, planktonic community. Other studies have shown that nutrient depleted cells can effectively transfer conjugative plasmids (Goodman *et al*, 1993) and that transfer into a wide range of indigenous bacteria is also possible (Fulthorpe & Wyndham, 1991).

Transfer by transduction and transformation have not been as extensively studied as conjugation, although with both these mechanisms transfer of chromosomal as well as plasmid genes have been studied. *In situ* transduction experiments have been confined to *Pseudomonas aeruginosa* in experimental enclosures. Transformation has been investigated in sediments, biofilms and in planktonic communities with three naturally competent bacterial genera. In all cases chromosomal genes transfer more readily than plasmids by transformation, with frequencies between 4×10^{-6} and 1×10^{-2} transformants per recipient (Fry *et al*, 1993).

The main conclusion from all these studies is that gene transfer occurs readily in aquatic environments. It also seems likely that a high proportion of the plasmids found in aquatic bacteria transfer broadly and will probably be major contributors to the gene flow occurring in nature. Perhaps gene transfer plays an important role in the plasticity of bacterial populations to environmental change. Studies of all types must continue until we can properly understand the role these transfer events play in the environment.

References

Bale, M.J., Fry, J.C. & Day, M.J. (1988) Transfer and occurrence of large mercury-resistance plasmids in river epilithon. Applied and Environmental Microbiology, 54, 972-978.

Fernandez-Astorga, A., Muela, A., Cisterna, R., Iriberri, J. & Barcina, I. (1992). Biotic and abiotic factors affecting plasmid transfer in *Escherichia coli* strains. Applied and Environmental Microbiology, 58, 392-398.

Fry, J.C. & Day, M.J. (1990). Plasmid transfer in the epilithon. In: Bacterial Genetics in Natural Environments (Eds. J.C. Fry & M.J. Day), pp. 55-80. Chapman and Hall, London.

Fry, J.C., Day, M.J. & Williams, H.G. (1993). Plasmid and chromosomal gene tranfer by transformation in the aquatic environment. In: DNA Transfer and Gene Expression in Microorganisms (Eds. E. Balla, G. Berensci & A. Szentirmai), p. 111-121. Intercept, Andover;

Fulthorpe, R.A. & Wyndham, R.C. (1991). Transfer and expression of the catabolic plasmid pBRC60 in wild bacterial recipients in a freshwater ecosystem. Applied and Environmental Microbiology, 57, 1546-1553.

Goodman, A.E., Hild, E., Marshall, K.C. & Hermansson, M. (1993). Conjugative plasmid transfer between bacteria under simulated marine oligotrophic conditions. Applied and Environmental Microbiology, 59, 1035-1040.

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Distribution of bacteria and their plasmids in deep freshwater sediments.

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Windermere, and other water bodies in the English Lake District, have provided a focal point for studies on the distribution of a range of prokaryotic and eukaryotic microbes. These include bacteria and protozoa in general (1,2,3) and their antibiotic resistance potential and distribution (4). More specifically, for example the distribution of *Cytophaga* sp.(5) and *Filibacter limicola* and *Peloploca* spp. has been examined (6,7). We have also considered the distribution of specific plasmids within a stream system (8). In addition, studies have addressed the survival potential of specific bacteria such as recombinant *Pseudomonas putida* (9) and *Aeromonas salmonicida* (10) in Windermere microcosms. The common feature to these studies is their two dimensional nature with respect to spatial distribution. None of these studies really address evolutionary aspects of either bacteria or their genetic systems. We have recently focused our research on the distribution of bacteria and their plasmids in deep freshwater sediments in the North Basin of Windermere. The sediments provide a chronological framework within which we can study the distribution and evolution of certain groups of plasmids and transposons.

The sediments lie below approximately 63 m at the deepest point (mean depth 25.1 m) and extensive characterization over the last 20-30 years has enabled us to correlate the depth of the core with time of deposition (11). It is estimated that the rate of sediment accumulation is approximately 2- 3 mm per year and core exhibits a distinct stratigraphy consistent with the time of sediment deposition. The North Basin core comprises a brown, non-compacted top layer between 0-2 cm, a black ooze from 2-30 cm and a brown mud from 30 cm - 5.5 m after which a distinct clay layer is apparent representing deposition in the late-glacial period of approximately 15,000 to 10,000 years ago. Extensive data exist on the chemical (water, carbon, nitrogen and mineral) content as well diatom, pollen and radiocarbon characteristics of the core from present day through to the glacial clay layers (11). In general, all these parameters are highly variable in the first 15 cm but show consistent degree of uniformity below this point. The microbiology of these cores, however, has largely been ignored with one exception; the ability of algae to survive deposition was examined by Stocker and Lund (12). Their analyses detected viable algae down to a depth of 25 cm in Windermere sediments which represented a time of deposition of 195± 12 years with a lower limit of 35 cm for all the lake sediments tested. It was

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inferred that the maximum depth at which the algae were detected could not be due to the burrowing activities of invertebrate populations alone.

We have adopted the deepest point (63 m) in the North Basin of Windermere as our primary sampling site for investigation. Sampling was carried out using a pneumatic Mackereth corer which recovers an 6 m intact sediment core from the lake bed. The core is hydraulically removed from the apparatus. Samples can be recovered aseptically at intervals of 50 cm from the centre of the core prior to processing and/or subsequent plating on appropriate solid media.

The aims of our preliminary analyses were to recover viable bacteria and total DNA from each sediment section in order to:

1) examine the distribution profile of culturable bacteria within the core;
2) examine the distribution profile of *Pseudomonas putida* using 16S RNA probes;
3) assess the presence of plasmids in recoverable bacteria;
4) examine the distribution of broad-host-range (IncP, N,and W) plasmids.

This presentation will discuss some of the results obtained.

References.

1. Jones, J.G. 1970. Journal of Applied Bacteriology 33, 679-687.
2. Jones, J.G. and Simon, B.M. 1975. Journal of Applied Bacteriology 39, 317-329.
3. Guhl B. and Finlay, B.J. 1993. FEMS Microbiology letters 107, 313-316.
4. Jones J.G, Simon, B.M., Gardener, S. and Pickup, R.W. 1986. Journal of Applied Bacteriology 60, 443-452.
5. Simon, B.M. and Jones, J.G. 1992. Freshwater Forum 2, 201-211.
6. Maiden, M.J and Jones, J.G. 1984a. Journal of General Microbiology 130, 2943-295.
7. Maiden, M.J. and Jones, J.G. 1984b. Archives of Microbiology 140, 44-49.
8. Pickup, R.W. 1989. Microbial Ecology 18, 211-230.
9. Winstanley, C., Morgan, J.A.W., Pickup, R.W. and Saunders, J.R. 1991. Applied and Environmental Microbiology 57, 1905-1913.
10. Morgan, J.A.W., Rhodes, G. and Pickup, R.W. 1993. Applied and Environmental Microbiology. 59, 884-880.
11. Pennington, W. 1973. Freshwater Biology 3, 363-382.
12. Stocker, J.G. and Lund, J.W.G. 1970. Limnology and Oceanography 15, 41-58.

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THE OCCURRENCE AND ROLE OF SELFTRANSMISSIBLE PLASMIDS IN SOIL AND RHIZOSPHERE.

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The objectives of our long-standing research in this area have been to gain insight into the natural gene flow, via conjugation, between bacteria in soil and rhizosphere, and to evaluate the capacity of soil populations to mobilize genes from introduced organisms into indigenous microbes.

An IncP1 broad host range self-transmissible plasmid, RP4p, has been shown to spread from an introduced *P. fluorescens* strain into various members of the indigenous bacterial community of the wheat rhizosphere in various different soil types. Furthermore, a mobilizable derivative of the IncQ plasmid RSF1010, pSKTG, was shown to be mobilized, by helper plasmid RP4p present in the original strain or in a co-introduced helper strain, into various indigenous bacteria. Transfer of pSKTG was not found in the absence of a co-introduced IncP1 helper plasmid.

To further analyse the putative presence of mobilizing (helper) plasmids in the bacterial communities of the wheat rhizosphere, two methods for plasmid isolation from these natural communities were applied, i.e. triparental mobilization isolation and exogenous isolation using resistance to Hg as a marker. Both methods resulted in the detection of plasmids with molecular sizes ranging between 40 and 80 kb. Plasmids were isolated at higher frequency from soil previously treated with Hg as compared to the corresponding untreated soil. A screening for markers on the plasmids obtained revealed the presence of chloramphenicol and streptomycin resistance determinants on some. The plasmids obtained are being characterized with regards to the presence of the *mer* operon (using PCR with primers of conserved regions), of *ori*T, of the IncP1 *trf*A gene and of various Inc determinants. Several of the plasmids obtained were transferable to suitable recipient strains with extremely high frequencies, and the putative role of these in adaptation of the rhizosphere microbial communities is being investigated using transfer and molecular approaches.

The results obtained will be discussed in the light of the possible occurrence of new plasmid types in soil, and strategies for detecting and studying these.

References:

Lilley, A.K., Bailey, M.J., Day, M.J. and Fry, J.C. (1992) Natural transfer plasmids from sugarbeet rhizosphere. In: Abstracts of the Sixth International Symposium on Microbial Ecology, 6-11 sept. 1992, Barcelona.

Selvaratnam, S. and Gealt, M.A. (1992) Recombinant plasmid gene transfer in amended soil. Wat. Res. Vol. 26: 39-43.

Smit, E., Van Elsas, J.D., Van Veen, J.A. and De Vos, W.M. (1991) Detection of plasmid transfer from *Pseudomonas fluorescens* to indigenous bacteria in soil using phage ΦR2f for donor counterselection. Appl. Environ. Microbiol. 57: 3482-3488.

Smit, E., Venne, D. and Van Elsas, J.D. (1993) Effects of cotransfer and retrotransfer on the mobilization of a genetically engineered IncQ plasmid between bacteria on filters and in soil. Appl. Environ. Microbiol. 59: 2257-2263.

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Exogenous isolation of conjugative plasmids from environmental sources.

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Exogenous plasmid isolation was used to assess the presence of IncQ-plasmid mobilizing functions in soil, active sludge and compost. Therefore, triparental matings were performed with *E. coli* (gamma-Proteobacteria) as a donor of an IncQ plasmid carrying the czc genes coding for resistance to the heavy-metals cobalt, cadmium and zinc. Zinc-sensitive *A. eutrophus* (β-Proteobacteria) were used as recipient and the indigenous microorganisms of the environmental samples were used as helper cells. The number of transconjugants isolated after such exogenous isolations could be use as a parameter to quantitate the IncQ-mobilizing potential of the environmental samples. IncQ-mobilizing activity was found in compost, soil and active sludge. Gel electrophoresis analysis of transconjugant lysates showed the presence of additional, large (about 60 kb) plasmids. Further mating experiments confirmed that the presence of these plasmids was essential for IncQ-plasmid mobilization. Some of the mobilizing plasmids so-isolated hybridized with IncP replication probes. Other such plasmids did not show cross hybridization with either IncP, IncN, IncQ, IncW, IncT, IncX, IncU, IncF, IncHI1, IncHI2 or IncL/M replication probes. The further genetic and molecular characterisation of the broad-host range mobilizing plasmids is in progress.

Bi-parental exogenous isolations were also performed. Extracts of heavy-metal polluted soil were mixed with zinc-sensitive *A. eutrophus* cells. Transconjugant *A. eutrophus* cells that had taken up zinc resistance genes from the soil microbial population were detected on selective plates. Further mating of the transconjugants with *A. eutrophus* showed that the zinc resistance genes are present on conjugative plasmids (transfer frequency 10^{-2} transconjugants / recipiënt). Transconjugants were found to contain plasmids of about 200 kb and carried the czc genes as shown by PCR analysis. Studies to determine the host range of the zinc conjugative plasmids are in progress.

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IV. Selected Talks from Posters

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The replication protein of the replicon pPS10 is a host range factor.

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pPS10 is a plasmid isolated from *Pseudomonas savastanoi* which is able to be established efficiently in *Ps. aeruginosa* but very inefficiently in *Escherichia coli* under the same conditions (37°C). Incubation of *E. coli* cells after transformation at low temperatures (30°C) increases the efficiency of the process.

After hydroxylamine treatment mutant plasmids able to be efficiently established in *E. coli* can easily be obtained. Three out of four mapped mutants show a C-T transition in the same position within the gene repA coding for the replication protein. The sequence in which the mutation is located corresponds to a putative leucine zipper motif in the corresponding protein. Since this motif has been described to cause dimerization of proteins our results suggest that molecular interactions of RepA either with itself or with with host proteins are important in host determination.

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The amino-terminal region of R388 TrwC has *oriT*-specific endonuclease activity and catalyzes conjugation-independent, site-specific recombination at *oriT*.

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The transfer region of the promiscuous IncW plasmid R388 (TRA_W) is comprised in a contiguous DNA segment of about 15 kb (Bolland *et al.*, 1990). Three R388 proteins, TrwA, TrwB and TrwC, are responsible for DNA mobilization. The deduced amino acid sequence of TrwC (Llosa *et al.*, 1994) shows in its amino terminal region conservation of a set of domains shared by a family of DNA relaxases (Ilyina and Koonin, 1992), while the carboxi terminal region contains a set of domains conserved in a family of DNA helicases (Hodgman, 1988).

Purified TrwC has *oriT*-specific endonuclease activity: it relaxes *in vitro oriT*-containing supercoiled plasmid DNA in the presence of Mg^{2+} and after incubation with SDS. Sequencing of the relaxed DNA shows a unique site where the bottom strand is interrupted. The *nic* site suggests that TrwC and TraI of plasmid F form a group of DNA-nickases different from the IncP family described in Waters and Guiney, 1993.

We have developed a system to test intramolecular *oriT*-specific recombination as an *in vivo* test for *oriT*-specific endonuclease activity. We have constructed a series of mutants in *trwC* and found that the 273 first amino acids of TrwC are enough to promote this recombination. The recombination events occur in the absence of conjugation, raising the possibility that TrwC is able to nick both strands of *oriT*. In this context, it is probably significant that TrwC is also able to mediate inter-molecular site-specific recombination at *oriT*.

In an attempt to separate the *oriT*-endonuclease and helicase activities of TrwC, we have cloned separately the amino- and carboxy- terminal regions of the protein in an expression vector; both peptides can be widely overproduced and purified. Our present aim is to assay their activities separately *in vitro* and use these gene constructions to perform a detailed genetic analysis of their molecular activity.

References:

Bolland, S., M. Llosa, P. Avila, and F. de la Cruz. 1990. J. Bacteriol. 172, 5795-5802.
Hodgman, T.C. 1988. Nature 333, 22-23.
Ilyina, T.V., and E.V. Koonin. 1992. Nucl. Acids Res. 13, 3279-3285.
Llosa, M., S. Bolland, and F. de la Cruz. 1994. J. Mol. Biol. 235, in press.
Waters, V.L. and D.G. Guiney. 1993. Mol. Microbiol. 9, 1123-1130.

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Plasmid stabilization by the *parA* system of plasmid R1

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The *parA* system of plasmid R1 mediates an approx. 100 fold stabilization of low copy number plasmids. Indirect evidence indicates that the *parA* system is a true partitioning system, that mediates plasmid stabilization by actively distributing plasmid molecules to the daughter cells at cell division. The *parA* system consists of three components: The *trans*-acting proteins ParM of 36 kDa and ParR of 13 kDa, and the *cis*-acting centromere like site of 160 bp, denoted *parC*. The *parM* and *parR* genes are co-transcribed as an operon from the *parA* promoter. The *parC* site, which contains the *parA* promoter flanked by 2 sets of 5 eleven bp direct repeats (the ParR boxes), is located upstream of *parM*. We can assign the following functions to the region encompassing the *parC* site: i)A plasmid carrying the *parC* site can be stabilized by donation of the two proteins *in trans*. Both proteins are required for maximal stabilization. ii) The *parC* site exerts incompatibility when present in high but not at low copy number. This incompatibility determinant is denoted *incA*. iii) The *parC* site encompasses the *parA* promoter. In steady state the promoter is severely down-regulated by ParR (more than 100 fold), whereas the ParM protein has no effect, neither when donated alone or together with the ParR protein. Binding of ParR to the *parC* region has been confirmed by gel shift and footprinting analysis.

Truncated *parC* sites, in which some of the direct repeats are deleted, can only be partially stabilized by donation of the two proteins *in trans*. Likewise do truncated *parC* sites not express incompatibility towards plasmids stabilized by full-length *parC* sites. This indicates that all repeats are required for proper functioning of *parC* in the partitioning process. However, since some of the truncated promoter fragments (*parC* sites) exhibit wildtype regulation by the ParR protein, only some of the direct repeats are required for regulation of the *parA* promoter.

By employing different *lacZ* fusions, we have found, that a region downstream of the *parA* promoter enhances transcription approx. 30 fold, rendering the *parA* promoter a very strong promoter. The responsible genetic element is preliminary termed *hex* (**h**igh **ex**pression element). A deletion analysis show that the *hex* site is situated downstream of the *parA* promoter and overlaps with the Shine-Dalgarno sequence of the *parM* gene. We are currently investigating the mechanism by which *hex* leads to promoter activation and whether other promoter-proximal elements are required for activation.

We have suggested a model of the function of the *parA* system, which takes in

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account the lack of incompatibility at low copy number and the high capability of the *parA* promoter:

Through most of the cell cycle, a tight nucleo-protein complex between *parC*, ParR and possibly ParM and host factors exists. When the plasmid is replicated, the complex is disrupted when the replication fork passes the structure. If the *parC* sites in the newly replicated plasmids are rebound by ParR before the plasmids are separated, then two *parC* sites saturated with ParR are in close proximity, and the two newly replicated plasmids will pair with a high probability ("twin-pairing"). The paired plasmids are the substrates in the partitioning process, which leads to distribution of the plasmids to the daughter cells, possibly via interaction with host encoded elements.

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CHARACTERIZATION AND MOLECULAR ANALYSIS OF THE BROAD-HOST-RANGE RepA/c REPLICON

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Plasmids from some incompatibility groups were described to be able to replicate and maintain in different host bacteria. Like all Inc P or Inc Q plasmids, the Inc A/C plasmids of Enterobacteriaceae, which maintenance functions remain to be studied, present a broad host range. Indeed, these plasmids are equivalent to the Inc P3 group in Pseudomonas sp like plasmids of the Inc P and Inc Q groups are equivalent to the Inc P1 and Inc P4 groups respectively in Pseudomonas sp.

In view of their large host range, these groups are of considerable interest both as natural vectors for disseminating genetic information among diverse species, and as cloning vehicles. That is why we have cloned the replicon of the Inc A/C plasmid RA1. An autoreplicative region of 2.79-kb, named RepA/C, was isolated. This region was able to generate replication in the two types of hosts (Enterobacteriaceae and Pseudomonadaceae), proving that all the necessary information for replicating in the different hosts was contained on the 2.79-kb RepA/C fragment.

A non-autoreplicative fragment of RepA/C (1.6-kb) was used as a probe and showed specific hybridizations with the Inc P3 - A/C plasmids from Pseudomonas and Enterobacteriaceae. Thus, this RepA/C replicon can be used as an epidemiological tool to probe for these broad host range plasmids.

The sequence determination and the first molecular analysis of the replicon RepA/C suggest that its general organization resembles that of the replicons controlled by the iteron mechanism *i.e.* an origin of replication (*ori*) with a succession of 13 direct repeats and an ORF coding for an essential replication protein (Rep). Interestingly, this iteron mechanism seems to be particulary adapted to plasmid replication, and distributed among many plasmids belonging to different incompatibility groups.

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CLONING AND SEQUENCE ANALYSIS OF PLASMID ORIGIN (*ori*) SEQUENCES FROM *RHIZOBIUM LEGUMINOSARUM*

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Rhizobium species form symbiotic relationships with the roots of leguminous plants, which are characterised by the formation of root nodules. The bacteria within the nodules reduce atmospheric nitrogen to ammonia which is assimilated by the plant. The genes essential for nodule formation and nitrogen fixation are generally located on large, low copy number plasmids (pSym). Other large cryptic plasmids are also usually present in *Rhizobium*; the number and size of these plasmids varies between strains. The replication of these large plasmids is tightly controlle and it seems plausible that sequences associated with their replication (*ori* & *rep*) could form the basis of plasmid-type specific probes.

The method of Mozo *et al.* (1990, Plasmid **23**:201-215) is being used to isolate sequences associated with plasmid replication. *R. leguminosarum* strain 3841 carries six endogenous plasmids, total DNA isolated from this strain has been used to construct an *Eco*RI library in pSUP202 in *Escherichia coli*. The vector pSUP202, is not stably maintained in rhizobia and carries *mob* functions that enable it to be conjugated from *E.coli* into *R.leguminosarum* strains. Conjugation of this library into *R. leguminosarum* 3841 generated more than 1 000 tetracycline resistant transconjugants. To date, 360 of the transconjugant strains have been screened for the presence of free pSUP202 derivative plasmids. An isolate has been identified, from which plasmid DNA can be re-isolated into *E.coli* and in which plasmid DNA can be detected by Southern hybridisation using a pSUP202 probe.

Data concerning the isolation of this plasmid (designated pST3) and the full extent of sequence determined to date will be presented.

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Determination of the basic replicon of the *Chromohalobacter marismortui* ATCC 17056 plasmid pCM.

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Moderately halophilic eubacteria are, together with the extremely halophilic archaea or halobacteria, the most important groups of microorganisms adapted to live optimally in hypersaline environments. Since moderately halophilic eubacteria can grow in a wide range of salt concentrations, they are an interesting microbial group from an ecological and biotechnological point of view. However, scarce information is available with respect to their genetics. Recently, our research group has initiated some genetic studies on representative moderately halophilic species in order to construct cloning vectors for this type of bacteria. We have isolated for the first time a plasmid, named pCM, from the moderately halophilic eubacterium *Chromohalobacter marismortui* ATCC 17056. This plasmid has been characterized by restriction endonuclease analysis and the corresponding restriction map has been constructed. The plasmid is 17.5 kb in size.

Using the suicide delivery properties of the R6K-based plasmid pGP704 a basic replicon was located in a 1.6 kb fragment and was also shown to replicate in other moderate halophilic bacteria like *Halomonas elongata* and *Volcaniella eurihalina*, failing to replicate or to be maintained in *Vibrio costicola* and *E. coli.* The sequence determination of this fragment and the comparison with other known replication origins are in progress.

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TRACKING RECOMBINANT ORGANISMS IN THE ENVIRONMENT: USE OF EUCARYOTIC LUCIFERASE GENES FOR DETECTION OF GENETICALLY ENGINEERED BACTERIA.

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The recent development of molecular detection techniques has greatly increased the ability to track microorganisms and engineered genetic markers in natural environments. Luminescence based techniques offer many of former methods and add the potential for in situ, nonextractive detection of marked cells in ecological samples. The genes coding for bacterial luciferases, *luxAB*, have been intensively applied for monitoring genetically engineered microorganisms. The eucaryotic luciferase genes have been less used for microbial detection. In their luminescent reaction, eucaryotic luciferases provide more efficiency and less energetic cost than bacterial luciferase. In addition, because of their eucaryotic nature and thus, presumably absent in all bacteria, since they are suitable to be detected by the most sensitive technique known for bacterial detection, PCR. This new genetic material introduces the possibility to distinguish bacterial population not only by its ability to emit light but also by the color of the light emited.

Different genetic construction were developed to apply eucaryotic luciferase genes for bacterial detection. The firefly and click beetle luciferase genes, *luc* and *lucOR* respectively, were cloned under constitutive and regulated control from different transcriptional units, *P1*, P_R and P_{trc} promoters. Comparison of the expression of each gene in *E.coli* from identical promoters, showed that bioluminescence produced by *luc* could be detected luminometrically in a more sensitive manner. In contrast, luminescence by intact *lucOR* expressing cells was much more stable and resistant to high temperatures than those with *luc*.

A system for stable tagging of gram negative bacteria with the firefly luciferase was developed. Stable integration into the bacterial genome was achieved by use of mini-Tn*5* delivery vectors. The system permitted the detection of tagged bacteria in the presence of more than 10^5 CFU per plate without the use of any selective markers. The firefly luciferase tagging system is an easy, safe and sensitive method for the detection and enumeration of bacteria in the environment.

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The soil insect *Folsomia candida* mediates a highly efficient interspecies gene transfer of recombinant plasmids by transformation

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We investigated the influence of the soil insect *Folsomia candida* on horizontal gene transfer of two small, recombinant plasmids. *F. candida* is a euedaphic, wingless soil insect, which feeds on decaying organic material (1). Adult specimen reach a length of 2 mm. Two donors were used in the experiment: a) the gram positive soil bacterium *Corynebacterium glutamicum* with the broad host range shuttle vector pUN1 (6.8 kb), a derivative of pUC18 and pUL330, and b) *Saccharomyces cerevisiae*, which harbours the *E. coli* - *S. cerevisiae* shuttle vector p707 (8.6 kb), a construct from pBR 322 and the 2μ yeast plasmid. Potential recipient strains tested were streptomycin resistant mutants of 11 soil bacteria and *E. coli* HB 101, respectively.

The standard experimental setup consisted of petri dishes, layered with water agar. Two 1 cm^2 nutrient agar slices (6 cm apart) were inoculated with donor and recipient cells (2×10^8), respectively. *F. candida* (50 specimen) were released into the model system and incubated at 20 °C for up to 22 h at 20 °C. The petri dishes were washed to recover the microorganisms and the faecal depositions of *F. candida*. Wash solutions were plated out onto double selective media and a subsequent colony hybridization of double resistant colonies with a specific 174 bp gene probe was carried out to detect horizontal gene transfer. Hybridization positive colonies were subjected to RFLP analysis to confirm the uptake and possible rearrangements of the plasmid DNA. The following controls were included in the experiments: a) samples with donor and rezipient, but without insects, to characterize the possibility of transfer during the washing procedure, b) samples with insects, but without recipient, to investigate the influence of microorganisms present in the gut of *F. candida*, and c) over night cocultivations of donor and recipient cells at 30 °C in LB medium to determine the gene transfer rate under optimal conditions.

The results have shown, that gene transfer was exclusively detected in the presence of *F. candida*. No control experiment exhibited transfer of the plasmid pUN1 into recipient cells. Cocultivations revealed transfer of pUN1 into recipient cells with all recipient strains, that gave rise to transformants in experiments with *F. candida*, except for *Agrobacterium radiobacter*, which was only transformable in the presence of the soil insect. The calculation of gene transfer rates were determined with donor inoculum densities. Comparison of these transfer rates in cocultivations and incubations with *F. candida* showed higher transfer rates in incubations with the soil insect ranging from 3.7 fold (*C. glutamicum*) up to 10^4 fold (*E. coli* HB 101). Both gram positive (*Arthrobacter citreus, A. globiformis, C. glutamicum*, and *Rhodococcus terrae*) and gram negative bacteria (*Agrobacterium radiobacter, Pseudomonas putida* and *E. coli*) were transformed with *C. glutamicum* pUN1. The transfer frequencies decreased, when less animals were included in the experiment. The minimal donor cell concentration that led to transformants in our experimental setup was 10^5 cells. First transformants were detected 8 h after *F. candida* was released into the model system. Plasmids were taken up without rearrangement or change of size. Experiments with heat inactivated donor cells of *S. cerevisiae* p707 and four different viable recipient strains gave rise to similar transfer efficiencies as with viable donor cells (10^{-6} per donor). Plasmid solutions of p707, applied onto faecal depositions, revealed transfer frequencies 10^3 fold to 10^4 fold lower than frequencies obtained with viable or heat inactivated

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cells. In an experiment with *S. cerevisiae* p707 as donor and *P. putida* as recipient, in which faecal depositions of *F. candida* were separated from the standard experiment and analyzed separately, transfer rates of 10^{-2} transformants/donor were observed. Feeding experiments with *F. candida* and different soil bacteria revealed feeding preferences of the soil insect. While *P. putida*, *Rhizobium trifolii* and *Curtobacterium* sp. were fed on readily, *Bacillus subtilis* or *A. citreus* were only taken up at much lower rates.

The data showed the influence of *F. candida* on horizontal gene transfer of recombinant plasmids. The transfer was very efficient (up to 10^4 transformants per µg plasmid DNA), compared to standard *in vitro* transformations with *E. coli* HB101 (10^6 transformants per µg plasmid DNA). The mechanism is considered to be an artificial transformation (2, 3) for three reasons: 1) the recombinant plasmids employed were free of any conjugative or mobilizable elements; 2) *E. coli* HB101 could be transformed, which is known for its inability of natural transformation; and 3) plasmid solutions gave also rise to transformants. The comparison of transfer rates of viable/heat inactivated donor cells and application of free plasmid DNA solutions suggested, that cell to cell contact is an important factor for the efficiency of gene transfer. The separation of faecal depositions from the experimental setup has shown, that competence of recipient cells must have been induced in the gut or later in the faecal depositions. Although no data are available about the specific components, that lead to competence of the recipient strains, we hypothesize, that a combination of mechanical stress by the feeding action of the insect and a subsequent breakdown of cell wall components by enzymatic reactions in the gut of *F. candida*, as well as the density and close proximity of donor and recipient cells in the faecal depositions were responsible for the formation of transformants. The uptake of donor and recipient cells by *F. candida* is essential for transformant formation and the concentration of cells in the gut or faeces of the insect determines the transfer efficiency. Therefore, the differences in transfer rates may in part be due to the feeding preferences of *F. candida*.

The data demonstrate the significance of the participation of soil animals in the transport of genetically engineered microorganisms and in horizontal gene transfer of recombinant plasmid DNA to soil indigenous microorganisms.

Literature

1 Borkott, H., and H. Insam. 1990. Symbiosis with bacteria enhances the use of chitin by the springtail, *Folsomia candida* (Collembola). Biol. Fertil. Soils 9: 126 - 129

2 Stotzky, G. 1989. Gene transfer among bacteria in soil; in: Gene transfer in the environment, S. B. Levy, R. V. Miller ed.: 199 - 205

3 Olson, B. H., Ogunseitan, O. A., Rochelle, P. A., Tebbe, C. C. and Y. L. Tsai. 1990. The implications of horizontal gene transfer for the environmental impact of genetically engineered microorganisms, in: Risk Assessment in Genetic Engineering, M. Levin, H. Strauss, ed.: 169 - 172

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QUANTITATIVE ASPECTS OF PLASMID BEHAVIOUR

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All the essential genetic information in bacteria is located in one double-stranded DNA molecule, the chromosome. (Some bacteria seem to have a linear chromosome and a few cases of bacteria with two chromosomes have been claimed to exist). Many bacteria also contain other replicating DNA molecules that live in harmony with their hosts - plasmids. Most plasmids seem to be circular but also linear plasmids exist. The size of the chromosome of bacteria such as *Escherichia coli* and *Bacillus subtilis* is about 5 Mbp, whereas plasmids range in size from 1.5 kbp to Mbps.

Plasmids replicate independently of discrete events in the cell cycle of the host. They control their replication and other processes that secure their maintenance in a bacterial population (partition, site-specific recombination, killing of plasmidfree hosts, and inhibition of cell division at low copy numbers).and their spread to other bacteria.

Plasmids measure their concentration and adjust the replication frequency to attain and retain a given copy number, which is a function of the plasmid, the host, and the growth conditions and can be as low as one and as high as hundreds or thousand copies per cell.

Plasmid systems actively partition the plasmid molecules between the daughter cells. This involves a centromer analogue, a site that is active *in cis* and, often, two proteins that can act *in cis* as well as *in trans*. Studies of incompatibility indicate that plasmid molecules pair during partition. In the absence of the *par* region, some plasmids are distributed randomly between the daughter cells: about 10^{-2n} of the daughters do not receive a plasmid (n is the average copy number of a newborn cell). A n value of 10 gives a loss frequency of about 10^{-6}, which is close to the resolution in stability studies. Other Par⁻ plasmids seem to be lost with a frequency that is worse-than-random. This might be due to a broad copy-number distribution or to some kind of clumping/aggregation causing the unit of segregation to be more than one plasmid genome.

Plasmids recombine to yield dimers, trimers, etc. The resolution of these into monomers is slow. Many plasmids encode site-specific-recombination (*ssr*) functions that resolve the multimers.

At least two types of systems kill from within those daughters that are born without a plasmid copy, one consisting of two proteins, a toxic protein and a protein that binds and inactivates the toxic protein (*e. g.* the Ccd system of plasmid F), and one in which a messenger for a toxic protein is posttranscriptionally inactivated because it binds an antisense RNA (*e. g.* the Hok/Sok system of plasmid R1).

Most plasmid maintenance experiments have been performed with populations growing exponentially in batch cultures. The basic replicon of plasmid R1 (n = 3-4) is lost in a frequency of about 10^{-2} per cell division. The frequency is reduced hundredfold if either the *par* or the *hok/sok* systems are added to the basic replicon; the presence of both systems gives a

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frequency of about 10^{-6}. The natural R1 plasmid is lost in a frequency $\leq 10^{-7}$. The difference might be due to Ssr or to the fact that R1 carries two killer systems, *hok/sok* and *parD*.

Plasmids often reduce the growth rate of their hosts. This is causes an accelerating loss of the plasmid from a plasmid-containing population - in the early phase, the plasmidfree cells are formed by loss of the plasmid, whereas at later stages, the growth-rate difference dominates.

Most stability experiments have been performed with populations growing exponentially in batch cultures, whereas in Nature bacteria presumably go through phases of rapid growth intersected by stationary conditions ("feast and famine"). Few studies have been devoted to plasmid maintenance in the postlogarithmic and stationary phases. In Nature, many bacteria grow on surfaces rather than in suspension. Much less is known about the quantitative aspects of plasmid maintenance during growth on surfaces than in liquid cultures.

Natural plasmids often constitute a few percent of the total DNA in their hosts; two percent is about 100 Mbp per genome equivalent of *E. coli*. Hence, a large plasmid is only present in a few copies per cell, wheras a small one is present in many copies. The DNA as such seems to limit the growth of the host - as the DNA content increases, the growth rate decreases and at a DNA content that is threefold higher than the normal amount, the growth rate becomes zero.

Many plasmids transfer themself by conjugation. A transfer system occupies about 20 kbp of genetic information. Several *tra* systems exist. The key features are sex pili that establish contact between donor and recipient. Some *tra* systems function best when the bacteria are growing on surfaces whereas others may cause efficient transfer in liquid medium. Most *tra* systems seem to be repressed and the frequency of transfer is fairly low. However, a newly acquired *tra* plasmid can rapidly transfer itself to other bacteria because there is a transient period of a derepressed state. The ability of transfer is of great ecological and evolutionary importance.

Many plasmids possess functions that kill related bacteria by bacteriocins produced from plasmid genes. The producing cell is immune to the bacteriocin.

Plasmids may recombine with other replicons. The resolution of the cointegrates may follow a different route compared to their formation and result in the formation of new combinations of genes. This is of great importance both ecologically and evolutionary (horizontal evolution) and may be an important factor in the evolution of genomes.

Reference

K. Nordström and S.J. Austin. Annu. Rev. Genet. 23 (1989) 37-69.

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V. Clinical and Environmental Plasmid-Mediated Gene Transfer

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Population selective amplification (PSA) and the evolution of plasmid-mediated β-lactamases

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An unexpectedly rapid evolution of plasmid-mediated β-lactamases is occurring all over the world. The widespread "classic" genes encoding TEM-1 or TEM-2 enzymes, hydrolysing ampicillin, have been modified by sequential mutations, so that these enzymes are now able to inactivate third generation cephalosporins and monobactams, or to resist the inactivation by clavulanate or other β-lactamase inhibitors. The emergence of some of these new "extended spectrum β-lactamases" as enzymes carrying two or more aminoacid modifications, strongly suggest that the "first" mutational events produced a non relevant change in phenotype and remained undetected. Nevertheless, these "first" **cryptic mutations were indeed selected** by antibiotic use. Site-directed mutagenesis permitted us to reproduce in an identical genetic context these early TEM-mutants. In various cases, a single mutation leads to a minor increase in minimal inhibitory concentration (MIC) to antibiotics such as cefotaxime; for instance, the change to serine in position 164 of TEM-1 (now is called TEM-10) increases the MIC from 0.03 µg/ml to only 0.06-0.12 µg/ml. Is that a selectable resistance?. It has been frequently assumed that mutants exhibiting very "low level" resistance were unselectable, in clinical practice, considering the high antibiotic concentrations attainable during treatments. Nevertheless, at any dosage, antibiotics create concentration gradients resulting from pharmacokinetic factors such as elimination rate or tissue distribution. Most probably, bacterial populations in the human body are facing a wide range of antibiotic concentrations. Any antibiotic concentration is potentially selective if it is able to inhibit the basal susceptible population but not in the same extent the variant harbouring the modification leading to MIC increase. In other words, a **selective antibiotic concentration** (SAC) is such if the MIC of the susceptible population is exceeded, but not that corresponding (even if it is very close) to the variant population. If the MICs of **both** is surpassed, **no selection** takes place (in fact the wild-type predominant population tends to survive after the challenge) and the same is true if the antibiotic concentration is below the MICs of **both** populations. Therefore, the **selection of a particular variant may only occur within a very narrow range of concentrations.** The continuous variation of antibiotic concentrations may

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ressemble a tuning device which "select" a particular radio frequency (the signal is the "valley" concentration between MICs of susceptible and mutant strains) a particular emission (the mutant strain). Because of the natural competition of homogenic bacterial populations in a close habitat, the "signal" is amplified, and very small differences in MIC results in dramatic ("quantum-type") changes in the populations structure. This model was tested in mixed homogenic cultures of TEM-1, TEM-12 and TEM-10 populations (90:9:1 proportions). Results confirm a sharp **population selective amplification (PSA)** at particular antibiotic concentrations. The sensitivity of this PSA-test to extremely subtle changes of antibiotic concentration is very high: at a cefotaxime concentration of only 0.008 µg/ml, TEM-12 began to be clearly selected against TEM-1. The selection was not more detectable (predominance of TEM-1) at 0.06 µg/ml, a type of response close to **periodic selection.** Only at higher antibiotic concentrations, TEM-10 began to be selected over TEM-1. These results suggest that **every mutational event,** even with apparently small phenotypic change, **has a real chance to be selected by antibiotics.** This selection increases the possibility of plasmid spread and the therefore the possibility of acquisition of new mutations. The behaviour of rapid population replacements as a consequence of subtle changes in the environment ressembles those found in chaotic systems. Research in **molecular population ecology** should complement the studies of molecular genetics and molecular physiology of antibiotic resistance.

References

M.C. Negri, M.I. Morosini, E. Loza, F. Baquero. 1994. In vitro selective antibiotic concentrations of β-lactams for penicillin-resistant Streptococcus pneumoniae populations. Antimicrobial Agents and Chemotherapy, 38: - (January)

Baquero, F., M.C. Negri, M.I. Morosini, J. Blázquez. 1994. The role of selective antibiotic concentrations on the evolution of antimicrobial resistance. Alliance for the Prudent Use of Antibiotics (APUA) Newsletter, 12: (Spring Issue).

Baquero, F. Vicente, M.F., Pérez-Díaz, J.C. 1985. Beta-lactam coselection of sensitive and TEM-1 β-lactamase producing subpopulations in heterogeneous Escherichia coli colonies. Journal of Antimicrobial Chemotherapy, 15:151-157.

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Plasmid/transposon-mediated spread of glycopeptide resistance

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Plasmid-mediated resistance to vancomycin and teicoplanin was first detected in 1986. It became rapidly apparent that glycopeptide-resistant enterococci were phenotypically and genotypically heterogeneous (Table 1). Inducible resistance to high levels of vancomycin and teicoplanin (VanA phenotype) is, in most strains, mediated by self-transferable plasmids. The genes necessary and sufficient for expression of the VanA phenotype are carried by transposon Tn*1546* of 10,851 bp which encodes nine polypeptides (Fig. 1) that can be assigned to four functional groups: transposition functions (ORF1 and ORF2), regulation of vancomycin resistance genes (VanR and VanS), resistance to glycopeptides by production of depsipeptides (VanH, VanA, and VanX), and accessory proteins that may be involved in peptidoglycan synthesis, but are not necessary for glycopeptide resistance (VanY and VanZ). Tn*1546* is delineated by imperfect (36/38 bp) inverted repeats and is structurally related to transposons of the Tn*3* family. The putative transposase (ORF1) and resolvase (ORF2) of Tn*1546* are transcribed in opposite directions, as in Tn*3* and in related transposons. However, the deduced amino acid sequences of ORF1 and ORF2 display a higher degree of similarity with proteins encoded by genetic elements from Gram-positive bacteria that do not have a Tn*3*-like structure. Plasmid conduction experiments performed in *Escherichia coli* indicated that Tn*1546* transposes in a host deficient in general recombination. Transposition is replicative, leads to the formation of cointegrate plasmids, and generates a 5-bp duplication at the target site. Analysis of the insertion specificity of Tn*1546* revealed similarities with that of Tn*3*. Transposition was obtained at multiple loci of a small plasmid in *E. coli*. The 5 base pairs duplicated upon insertion had a high adenine plus thymine content. A putative "hot-spot", in which insertion of Tn*1546* occured in both orientations, was flanked by a tetranucleotide also present at the ends of the transposon. Cointegrate molecules generated by replicative transposition of Tn*1546* were found to be stable in *E. coli* hosts deficient in homologous recombination. This may imply that Tn*1546* does not code for a functional resolution system although, as already mentioned, ORF2 displays extensive similarity with various resolvases. Several lines of evidence suggest that high-level resistance to glycopeptides in enterococci isolated in Europe and North America is mediated by transposons similar to Tn*1546*. Nucleotide sequences related to the *vanA* gene were detected by DNA hybridization under stringent conditions in all representatives of a collection of 31 strains expressing the VanA phenotype. Further analyses of three strains revealed similarity with the entire sequence of Tn*1546* and a common gene organization. The sequences of the inverted repeats of these elements and of Tn*1546* were identical. Finally, the *vanR* genes of two strains isolated in France and in Spain are identical in sequence. High-level glycopeptide resistance has been detected in strains of *E. faecium*, *E. faecalis*, *E. avium*, *E. durans*, and *E. mundtii* belonging to various biotypes. These characteristics of strains with a VanA phenotype indicate that spread of high-level glycopeptide resistance is not due to an epidemic of a strain or a plasmid. In plasmid pIP816, where it was originally detected, Tn*1546* is inserted into a sequence nearly identical (96 %) to a streptomycin resistance gene and there is a 5-bp duplication of the target sequence. A Tn*1546*-related element was also flanked by 5-bp direct repeats in a different sequence environment. These observations indicate that transposition plays a role in dissemination of vancomycin resistance genes among enterococcal replicons. Conjugal transfer of various plasmids, that have acquired Tn*1546* or closely related elements by transposition, appears therefore to contribute to the spread of vancomycin resistance genes in enterococci.

Strains with genes of the *vanB* class are inducibly resistant to various levels of vancomycin (VanB phenotype; Table 1). These strains remain susceptible to teicoplanin although vancomycin induces resistance to this antibiotic. VanB resistance in certain strains is transferable by conjugation. Analysis of transconjugants by pulsed-field gel electrophoresis indicates that *vanB* is located on large, 90-240 kb, chromosomal elements.

E. gallinarum and *E. casseliflavus* are intrinsically resistant to low levels of vancomycin and susceptible to teicoplanin (VanC phenotype, Table 1). Vancomycin resistance is associated with sequences related to genes *vanC-1* that is specific of *E. gallinarum* or *vanC-2* specific of *E. casseliflavus* but the mechanism of resistance remains unknown. Strains of *E. gallinarum* and *E. casseliflavus* resistant to macrolide-lincosamide-streptogramin B-type (MLS) antibiotics and to high levels of glycopeptides (VanA phenotype) were isolated recently from the faeces of a patient with acute myeloïd leukaemia treated with oral vancomycin. High-level resistance to glycopeptides together with MLS resistance were transferable from the two clinical isolates to *E. faecalis*. The transconjugants were resistant to high levels of glycopeptides and MLS and harboured a plasmid,

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pIP218, of approximately 16 kb carrying the *vanA* gene cluster. Since *E. faecium* strains highly resistant to glycopeptides were first isolated in the blood cultures of the patient, it is likely that pIP218 originated in these strains. Transfer of pIP218 between the different enterococcal species probably occured by conjugation in the gastrointestinal tract as already observed for other resistances. The fact that strains of *E. gallinarum* and *E. casseliflavus* have acquired genes conferring high-level resistance to glycopeptides suggests that, under certain circumstances (*e.g.*, oral vancomycin therapy), constitutive low-level resistance to this drug is not sufficient for survival of the host bacterium.

Table 1. Glycopeptide resistance in enterococci

	Acquired resistance		Intrinsic resistance
Genotype	*vanA*	*vanB*	*vanC*
Phenotype	VanA	VanB	VanC
Vancomycin MIC (µg/ml)	64- >1,000	4- >1,000	2-32
Teicoplanin MIC (µg/ml)	16-512	0.5-1	0.5-1
Expression	inducible	inducible	constitutive
Genetic basis	plasmidic (Tn*1546*)	chromosomal	presumably chromosomal
Transferability	+	+	-
Species	*E. faecium* *E. faecalis* *E. avium* *E. durans* *E. mundtii*	*E. faecium* *E. faecalis*	*E. gallinarum* *E. casseliflavus*

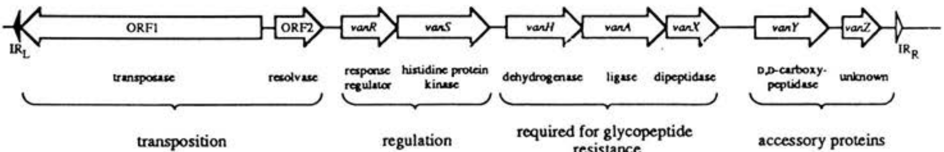

FIG. 1. Map of Tn*1546*. Open arrows represent coding sequences. Closed and open arrow heads labeled IR_L and IR_R indicate the left and right inverted repeats of the transposon, respectively. Genes *vanH*, *vanA*, and *vanX* are cotranscribed from a promoter located in the *vanS-vanH* intergenic region.

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THE pR PLASMID AS A TOOL TO INVESTIGATE THE MOLECULAR BASIS OF THE HOSPITAL INFECTIONS

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The aim of my tolk will be to demonstrate that (i) in the pR plasmid three genes, bat, uvp1 and mucAB, cooperate togheter in the interest of the plasmid genome maintenance and in the spreading in the hospital environement; (ii) the bat gene coordinates the expression of uvp1 and mucAB genes.
The pR plasmid is a derivative of plasmid R46, belonging to N-incompatibility group originally isolated from a hospitalized patient. The pR plasmid contains the mucAB genes and the recA activated protein regulates their expression (1,2). These genes are involved in the origin of spontaneous mutations and in the repair of DNA damage caused by UV light (frequently used for sterilization).
By using an artificial hybrid between phage lambda and pR plasmid, we have shown that the rep region of the pR plasmid encodes a function which regulates the expression of the mucAB genes (i.e. plasmid genes that are under the negative control of the lexA gene and are responsible for an encreased rate of spontaneous mutagenesis and resistance to UV and chemicals). By deletion mapping we have located the gene (termed bat) encoding the antagonist of lexA in the *rep* region of the pR plasmid. The pR bat gene function is essential for plasmid replication and for spontaneous induction of the SOS response in *Escherichia coli*. Mutations preventing single-stranded DNA production, needed for pR replication, also prevent the induction of the SOS system.
In addition to mucAB and bat genes, the pR also contains the uvp1 gene (3) that encodes a 20kDa protein which appears to be responsible for enhancement of both UV survival and recombination in *E.coli*. By using the purified Uvp1 protein, we have identified a DNA region which is specifically involved in Uvp1 binding; the binding region is located about 1 kb upstream from the uvp1 encoding sequence, within in a region between the ampicilin resistance gene (OxaII) and an open reading frame coding for an integrase (4,5). As previously reported by Martinez and de la Cruz (5), recombination events in this region elicit formation of cointegrates. The uvp1 function is to resolve the cointegrated plasmids. The resolution of cointegrated plasmids gives rise to different associations of the antibiotic resistences. The uvp1 present another new and interesting property: the regulation of the gene depends on the activated RecA protein.
In conclusion, the recA protein coordinates the expression of the uvp1 and mucAB genes each time that the pR plasmid replicates by rolling circle mechanism induced by the bat protein.

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References

1. Battaglia P.A., Gigliani F., Marcucci L. and Elli R. (1987). Mol. Gen. Genet **209**, 41-48.
2. Gigliani F., Ciotta C., Del Grosso M.F. and Battaglia P.A. (1993). Mol. Gen. Genet. **238**, 333-338.
3. Gigliani F., Sporeno E., Perri S. and Battaglia P.A. (1989). Mol. Gen. Genet. **218**. 18-24.
4. Stokes H.W. and Hall R.M. (1989). Mol. Microb. **3**, 1669-1683.
5. Martinez E. and de la Cruz F. (1990). EMBO J. **9**, 1275-1281.

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VI. Molecular Ecology

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"DETECTION OF MICROORGANISMS AND MONITORING OF THEIR METABOLIC ACTIVITY IN NATURAL ENVIRONMENTS"

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The bacterial taxonomist's dream must always have been to develop a staining method which specifically, and without isolation and purification of the bacteria, can identify unequivocally a species. (At least, this is my naïve vision of how taxonomists dream). The modern phylogenetic identification methods based on *in situ* hybridization of fluorescently labelled oligonucleotides complementary to species specific sequence domains in one of the large ribosomal RNAs fulfill this dream. The sequencing of a very large number of ribosomal RNA genes in different organisms has provided a unique database of information concerning evolutionary relationships, and the distribution of conserved and variable domains has made it possible to specifically design oligonucleotides which will only recognize members of kingdoms, phylo-groups, families, genera or species dependent on the particular sequence domain. Combined with fixation in paraformaldehyde and fluorescence microscopy the *in situ* hybridization of rRNA allows identification of single bacterial cells directly in environmental samples.

The ribosome is a key cellular component involved in the complex process of translation, and its synthesis requires a substantial amount of energy and resources; thus, at fast growth of bacteria the ribosomal proteins may constitute up to 25% of total cellular protein. It is therefore not surprising that the synthesis of ribosomal components is regulated and coordinated, and already in the early days of microbial molecular biology the famous experiments of Maaløe et al. in Copenhagen showed that the content of ribosomes in bacteria correlated with the growth rate of the cells: Fast growing cells contained many ribosomes, slow growing cells had fewer. This correlation seems to be true for most if not all bacteria. By growing cultures of purified strains in a number of different media supporting different growth rates a standard curve for this relationship may be obtained.

In a series of studies we have investigated a strain of *E. coli* and its colonization of and behaviour in the large intestine of mice. One important aim was to localize the bacteria relative to the different sectors in the gut, and another was to obtain estimates of the bacterial growth rates during the process of establishment and persistance. The strain we used was originally isolated from a rat, and a preliminary characterization of the strain showed that it grows very well in standard *E. coli* media, and it was shown to harbour two fairly large plasmids, which we have used as identification markers. A standard curve for the relationship between ribosome content and growth rate was produced in test tube experiments, and the outcome was as expected. A specific *E. coli* oligonucleotide probe labelled with a fluorescent dye was designed for hybridization experiments.

The colonization model applied in these experiments is based on a treatment of the mice with streptomycin in order to remove the aerobes including the enterics. Most anaerobic bacteria are not affected by this drug treatment. By feeding streptomycin resistant bacteria

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to the animals colonization will take place and may be monitored either from fecal samples or by thin sectioning of the digestive tract. Both types of samples can be analysed for their content of the fed bacteria through *In situ* hybridization followed by fluorescence microscopy. The *E. coli* strain we use was made streptomycin resistant and excellent colonization in the antibiotic treated mice was always obtained. No hybridization was observed in the animal samples prior to feeding with *E. coli* showing that the probe is specific. The microscopy of the *E. coli* infected mouse samples showed that this organism constituted from a few up to 15% of the total population, and they were exclusively found in the large intestine. Capturing microscopic images with a CCD camera provided the possibility to quantify the hybridization signals, which could be converted into estimates of ribosome contents by comparison with the standard curve; thus, estimates of growth rates based on hybridization signals from single cells were obtained.

The major conclusions so far obtained from this study are:

1) *E. coli* grows in the mucus of the large instestine of mice

2) The growth rates seem to be very fast initially, but even at late times after infection the bacteria grow surprisingly fast.

3) There is selection for slow-growing bacteria in the gut, which, however, may have a selective advantage in the absence of oxygen.

4) The method of identification and monitoring of metabolic activity is general and certainly not restricted to the presented case.

Key references:

Amman, R.I., Krumholz, L., and Stahl, D.A. 1990. J. Bacteriol. **172**, 762-770 (*In situ* hybridization)

Cohen, P.S., Arruda, J.C., Williams, T.J., and Laux, D.C. 1985. Infect. Immun. **48**, 139-145 (The mouse model)

Krogfelt, K.A., Poulsen, L.K., and Molin, S. 1993. Infect. Immun., in press (Part of the presented data)

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A NOVEL SYSTEM TO EXPLORE THE ECOLOGICAL/EVOLUTIONARY ROLE OF BARRIERS TO GENE DISPERSAL IN BACTERIA.

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The microbial world has developed effective mechanisms of gene transfer as well as equally effective mechanisms to inhibit gene transfer, which on one hand facilitate the rapid spread of advantageous properties when environmental conditions change, and on the other hand favour the conservation of genetic identity and stability. We present here a system that constitutes an interesting tool to explore the ecological and evolutionary consequences of shifting the equilibrium towards genetic constancy, for example under conditions which ordinarily select for change.

We have developed a gene containment system based on a lethal donation of a killing function. The system involves two genes, the kill gene, the one that codes for a lethal function, and the immunity gene, the one that codes for a specific repressor of the lethal function. In the genetically engineered microorganism (GEM), the gene specifying the lethal function is closely linked to the determinant of the novel phenotype, whereas the immunity gene is located distant from the recombinant genes such that co-transfer of immunity gene with kill gene is highly unlikely. Transfer of the determinant of the novel phenotype to a new organism will be accompanied by that of the lethal function but not by that of the immunity function, and the recipient organism will be killed. In a model such as this, the killing function should be active in as wide a range of potential recipients as possible, whereas the immunity function should exist naturally in a very restricted number of organisms. The colicin E3 system fulfils both requirements. Colicin E3 is a bacteriocin produced by certain colicinogenic strains of *Escherichia coli* which kills closely related microorganisms by inhibiting protein synthesis. It does this by specifically cleaving a fragment of about 50 nucleotides from the 3′ end of the 16S rRNA containing *inter alia* the mRNA binding site (Shine-Dalgarno site). Colicinogenic strains are immune to colicin E3 since they produce an immunity protein that binds to colicin E3 and prevents its action. Genes *colE3* and *immE3* , encoding colicin E3 and E3 immunity protein respectively, are present in a non self transmissible plasmid, pColE3-CA38, and they are cotranscribed in an operon.

We have cloned and constitutively expressed *colE3* and *immE3* genes separately. The

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immE3 gene was integrated and constitutively expressed in the chromosome of different *E. coli* and *Pseudomonas putida* strains. On the other hand, by using PCR techniques and *E.coli*ImmE3 as a recipient strain, the *colE3* gene was cloned under the control of the *Ptac* promoter in pVLT31, a promiscuous plasmid able to replicate both in Gram-positive and Gram-negative bacteria. We have checked the efficiency of our containment system by using different ways of DNA transfer, transformation and conjugation, and different recipient microorganisms representatives of the main subgroups of Proteobacteria: *E. coli*, *P. putida* , *P. fluorescens*, *Alcaligenes eutrophus*, *Comamonas acidovorans*, *Rhizobium meliloti* and *Agrobacterium tumefaciens*. In all cases we obtained between 10^4-10^6 times less plasmid-containing tetracycline-resistant recipients with the plasmid expressing colicin E3 than with the wild type vector.

These results show that colicin E3 and immunity E3 can be engineered as an efficient gene containment system, and that the bactericidal effect of colicin E3 is manifested in a variety of important strains for environmental purposes. Due to the fact that the sequence in the 16S rRNA cleaved by colicin E3 is absolutely conserved in all prokaryotes, this killing function should be universal in nature and an excellent candidate to design barriers that will decrease dispersal of recombinant genes among indigenous microorganisms of ecosystems into which a GEM is deliberately or accidentally introduced, an important aspect of risk assessment associated with the release of GEMs into the environment. The lethal gene donation system described here represents, in addition, a new strategy to study the ecological and evolutionary implications of shifting the natural equilibrium between gene transfer and gene constancy.

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Microbial sensing of aromatic pollutants : Specificity and cross-talk among catabolic promoters of *Pseudomonas*

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Aromatic pollutants, in particular alkyl- and chloro-aromatic compounds, are the most ubiquitous xenobiotic molecules present in virtually all ecosystems as the result of urban and industrial activity since early this century. Microbial communities have evolved in many cases the ability to cope with such molecules by assembling catabolic pathways which afford the bioconversion of aromatic structures into central metabolic intermediates. Although the rule is that complete mineralization of these compounds requires the concourse of enzymatic activities provided by different members of the community, there are many cases also in which one single strain can use as the sole source of carbon and energy an otherwise recalcitrant compound. Such pathways frequenty appear encoded by transmissible plasmids, sometimes included within large transposons, which under adequate pressure, may spread and amplify the capacity of a given community to metabolize these compounds. Many strains of *Pseudomonas* and related Gram-negatives carry such plasmids which allow the complete metabolism of a variety of unusual aromatic chemicals.

Analysis of DNA and protein sequences of the enzymes which form catabolic pathways has revealed that, as a rule, isoenzymes which catalyze similar or identical steps within catabolic pathways tend to be highly homologous even in cases where the substrates of the pathway are very different. Catechol 2,3 dioxygenases of the *meta*-TOL pathway, the lower NAH pathway, the *bph* pathway and the *dmp* pathway are extremely similar, even though they are integrated within catabolic operons targeted to the degradation of compounds as diverse as *meta*-toluate, salicylate, biphenyl and phenol. These homologies suggest a common evolutive origin for the enzymes and even for different portions of the pathways, which may get assembled at a given time to respond to a novel substrate through DNA slippage or other mechanisms. The mechanism of transcriptional control under which these operons occur, is however, extremely different regardless of the structural similarity between pathway inducers. The *meta*-NAH pathway, activated by salicylate, uses a transcriptional control system based on the NahR protein, which belongs to the LysR family of regulators. On the contrary, the *meta*-TOL pathway is regulated by an activator, XylS, of the AraC family. This is particularly striking, since XylS mutants can be easily found which respond to salicylate. This suggest that transcriptional control evolves quite independently of the structural genes of the catabolic operons. To gain some insight in the way control elements get assembled within biodegradative pathways we have examined in detail two cases in which a single promoter region located in front of a polycistronic operon can respond to at least two regulators which respond themselves to different aromatic effectors.

The first case is that of the *Pu* promoter of the archtypical TOL (toluene degradation) plasmid pWW0 of *Pseudomonas putida*. This is a sigma-54 dependent promoter which requires the binding of a cognate activator, XylR, to upstream activating sequences (UAS) in order to initiate transcription when cells are exposed to toluene. A distinct IHF sequence placed between the UAS

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and the RNApol binding site ensures not only a maximum activity under induced conditions, but also enhances the fidelity and the strictness of the response to the right inducer/activator. This effect is due to the DNA bending caused by IHF and can be mimicked by substituting the IHF site within the promoter by a statically curved DNA sequence. In spite of such barriers, *Pu* can also be illegitimately activated by the regulator of a totally different catabolic pathway, namely DmpR, of the phenol degrading pathway of plasmid pVI150 of *Pseudomonas* CF600. Both in the absence and the presence of XylR, DmpR makes *Pu* to respond to phenol by binding the very same UAS which are the target sites for XylR.

The second example is the XylS-dependent and independent activation of the *Pm* promotor of the TOL plasmid : The *Pm* promoter of the *meta*-cleavage operon carried by the plasmid becomes activated by the plasmid-encoded XylS regulator in the presence of a variety of benzoate effectors. XylS belongs to the AraC family of transcriptional regulators and acts upon *Pm* by binding a distinct directly repeated sequence within the promoter region. In the absence of XylS, *Pm* is still responsive to unsubstituted benzoate but with an induction kinetics and within a range of activity which differs substantially of the XylS-mediated activation. XylS-independent induction by benzoate is dependent on sigma-54 and regulated by growth-phase. However, XylS-dependent and independent transcription is initiated at the very same nucleotide. Furthermore, a series of deletions and mutations at the *Pm* promoter sequence showed the same overall pattern of responsiveness to benzoate with or without XylS, thus providing genetic evidence that the same promoter structure is recognized and activated by at least two different regulators. One of them is XylS, while the other, provided by the host bacterium, could be related to the chromosomally-encoded benzoate-degrading pathway.

All these data indicate that activation of catabolic promoters is not a very specific process. Unrelated regulators present in the same cell or acquired occasionally from other members of the community can make promoter sequences to shift totally its specificity and transcribe catabolic genes or operons in response to signals for which they do not seem to be initially programmed. This may further enhance the capacity of bacterial communities to quickly adapt to changing environmental conditions by circulating "regulatory cassettes" which can be recruited for the assembly of transcriptional control elements in new degradative pathways.

de Lorenzo, V., Herrero, M., Metzke, M. and Timmis, K.N. 1991. EMBO J. 10 : 1159-1167.

Harayama, S. and K .N. Timmis. 1989. In: Hopwood,D., Chater, K. (eds). Genetics of Bacterial Diversity. Academic Press, London, pp. 151-174.

Kessler, B.,V. de Lorenzo, V. and K. N. Timmis. 1992. Mol. Gen. Genet. 233: 293-301.

Kessler, B., V. de Lorenzo and K. N. Timmis. 1993. J. Mol. Biol. 230: 699-703.

Ramos, J. L., F. Rojo and K.N. Timmis. 1990. Nucl. Acids Res. 18: 2149-2152.

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POSTERS

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MOBILIZATION OF RSF1010 BY THE INCW PLASMID R388 AND THE INCP PLASMID RP4 REQUIRES THE CONJUGATIVE PILUS AND A TRAG-LIKE PROTEIN

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RSF1010 belongs to the plasmid incompatibility group IncQ and like many other non-conjugative plasmids only encodes DNA mobilization genes: oriT and Mob proteins. Therefore, it can only be mobilized when an appropriate conjugation system is provided by a coexisting conjugative plasmid.

In this work, the IncW plasmid R388 genes involved in mobilization of non-conjugative plasmid RSF1010 have been analyzed by genetic complementation in mating experiments. This mobilization requires the genes involved in pilus synthesis, plus trwB. Similarly, mobilization of RSF1010 by RP4 requires Tra2 plus traF and traG (Lessl et al.,1992) and the mobilization by this incompatibility group is extremely efficient. When trwB plus pil_w is substituted by traG plus pil_w, RSF1010 is mobilized at hight frequencies. In this case, traF is not necessary, even being essential for RP4 conjugation. Besides, traG can efficiently substitute for trwB in RSF1010 mobilization, but it is very inefficient at complementing selftransfer of R388 TrwB mutants. In order to check any difference in the efficiency of mobilization of different plasmids, the same studies were made with ColE1, and the results showed the same picture: the frequency of mobilization is very similar and the same genes are involved.

The functional complementation found in this work between proteins of different conjugative systems confirms the similarity shown by their aminoacid sequencies (Llosa et al.,1994). TrwB, TraG and VirD4, together with TraG of RP4 are possibly the mediators between the specific relaxosomes formed by the initial processing of the donor DNA and the core transfer apparatus, implicated in the extrusion of the DNA and suggests the existence of a protein family common to many DNA-transfer processes.

References:

Lessl,M., W.Pausegrau and E.Lanka. 1992. Nucl.Acids Res. **20**,6099-6110.

Llosa,M., S.Bolland and F. de la Cruz. 1991. Molec. Gen. Genet.**226**, 473-483.

Llosa,M., S.Bolland and F. de la Cruz. 1994. J. Mol.Biol. **235** (in press).

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Identification and characterization of the maintenance functions of the IncHI plasmids.

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Members of the IncHI group are large conjugative plasmids presenting an unusual thermosensitive transfer (Smith, 1973). We have isolated and characterized three maintenance regions of the IncHI1 plasmids: two replicons and a locus that expresses incompatibility toward plasmids of both IncHI subgroups (IncHI1 and IncHI2). By DNA-DNA hybridization experiments, we demonstrated that these maintenance regions are different and that they are specific to, and carried by, all IncHI1 plasmids tested. In view of this specificity we decided to designate the replicon isolated from R27 plasmid as RepHI1A and the replicon from pIP522 as RepHI1B (Newnham and Taylor,in press, 1993; Gabant et al., 1993 and in press). These two autoreplicative regions are not related to a third replicon present in all IncHI1 plasmids that bears homology with RepFIA and that expresses the characteristic incompatibility of IncHI1 subgroup plasmids toward F factor (Taylor et al. 1985 and Saul et al., 1988). The RepHI1A, RepHI1B and IncHI1 determinant were located on the R27 map and sequenced (Gabant et al., 1993, Newnham and Taylor, submitted). The DNA sequence of the IncHI1 region reveals five direct repeats of 31bp. We obtained evidence that it is most probably a component of an active partition locus involved in the maintenance of IncHI1 plasmids. Interestingly, this IncHI1 locus hybridizes only with IncHI1 plasmids but expresses incompatibility toward plasmids of both IncHI subgroups (IncHI1 and IncHI2).
The DNA sequence of RepHI1B reveals DNA repeats of 17 base pairs located upstream and downstream from a gene coding for a 32 kilodalton protein required for replication (RepA). Interestingly, RepA presents significant homology with other Rep proteins encoded by plasmids belonging to different Inc groups : P1(IncY), Rts1 (IncT), RepFIB (IncFI) and RepHI1A (IncHI1). The observed resemblances suggest that these replicons emerge from a common ancestor, despite their belonging to different incompatibility groups. All these results and a first molecular analysis of the RepHI1B replicon provide strong evidence that it belongs to the group of plasmids which control their copy number by an iteron mechanism.
Several members of the IncHI1 group are multiresistant plasmids of medical and epidemiological importance (for a review, see Taylor, 1989). Characterizing their maintenance loci will not only enhance our basic understanding of the replication and partitioning mechanisms, but also greatly facilitate identification of these plasmids, providing powerful tools for epidemiological studies.

-Gabant, P., Newnham, P., Taylor, D. and M. Couturier. 1993. J. of Bacteriology. Dec.
-Gabant, P., Ouazzani Chahdi, A. and M. Couturier. Plasmid. in press.
-Newnham, P. and D. Taylor. Mol. Microbiol., in press.
-Saul, D., Lane, D. and P.L. Bergquist. 1988. Mol. Microbiol. **2** : 219-225.
-Smith, H. R., Grindley, N. D., Humphreys, G. O. and E. S. Anderson. 1973. J. Bacteriol. **115**: 623-628.
-Taylor, D. E., Hedges, R. W. and P.L. Bergquist. 1985. J. Gen. Microbiol. **131**: 1523-1530.
-Taylor, D. E. 1989. In L. E. Bryan (ed), Handbook of experimental pharmacology. Springer-Verlag, Berlin, p. 325-357.

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AGROBACTERIUM-MEDIATED DNA TRANSFER WITHIN PLANT CELLS

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Agrobacterium tumefaciens is a gram-negative soil-born bacterium responsible for the crown gall disease of plants. Its main peculiarity resides in the release of particular genes located on the transferred DNA (T-DNA), which is part of the large tumour-inducing plasmid (pTi), and their further integration and expression in the plant-cell nucleus. To date it is believed that specific binding of the bacteria to the plant-cell surface precedes the mobilisation of a T-DNA/protein complex from the bacterial cell to the plant cell by a mechanism analogous to bacterial conjugation . One of the main open questions in this interkingdom interaction is how the T-DNA complex gets into the plant cell. By using a microinjection approach we show several novel features for T-DNA transfer : (i) Dispensability of the proposed bacterial attachment to the plant-cell surface. (ii) The process of DNA transfer itself, from the bacteria to the eukaryotic nucleus, can occur inside the plant cell. (iii) The transfer of T-DNA is absolutely dependent on induction of bacterial virulence (vir) genes. Hence, our results show that *A. tumefaciens* can function as an intracellular infectious agent in plants.

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ROLE OF σ^s IN MAINTENANCE OF THE PROMISCUOUS PLASMID pLS1 IN *Escherichia coli*

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The alternative sigma factor σ^s, encoded by *rpoS*, is responsible for the expression of a wide number of genes during the stationary phase of *Escherichia coli* (1). It is also essential for survival of bacteria in conditions of prolonged starvation, and a mutant allele of this gene confers adaptative advantages in stationary phase. This mutation occurs spontaneously in long-starved popullations of *E. coli* (2). Homologues of the *rpoS* gene have been recently found in *Salmonella choleraesuis*, being related to plasmid-mediated bacterial virulence (3).

We are investigating the possible control of plasmid pLS1 promoters by σ^s. pLS1 is a broad-host-range plasmid, capable of replicating in different Gram-positive and Gram-negative bacteria, including *Escherichia coli* (4). We have found that the number of copies of pLS1 depends on the *rpoS* background, at least in stationary phase. Both *rpoS*$^+$ and *rpoS*$^-$ strains present similar number of copies of pLS1 during exponential growth, but while in wild type strains copy number increases 10-fold in stationary phase, in *rpoS*$^-$ mutants, copy number remains unchanged. As a consecuence, pLS1 is lost more quickly in *rpoS* mutants when cultures are grown in the absence of selection.

Recently, we have found that σ^s-regulated promoters are located in intrinsically curved DNA regions (5). On the basis of this finding we have examined the role of σ^s in transcription from pLS1 promoters, as two of the promoters of pLS1 -the RNAII promoter ($P_{\alpha II}$) and the *copG repB* promoter (P_{cr}), both essential for plasmid replication and control of copy number- are located in curved DNA regions (6). Analysis of ß-galactosidase activity of $P_{\alpha II}$::*lacZ* and P_{cr}::*lacZ* fusions indicate a negative effect of σ^s upon $P_{\alpha II}$, and apparently no effect upon P_{cr}. Wether σ^s controls directly or indirectly pLS1 copy number remains to be determined.

All these results, and their implications in plasmid maintenance will be discussed in this poster.

REFERENCES

1 Kolter, R. *et al.* 1993. *Ann. Rev. Microbiol.* (in press)
2 Zambrano, M.M. *et al.* 1993. *Science* 259, 1757-1760
3 Fang, C.F., *et al.* 1992. *Proc. Natl. Acad. Sci. USA* 89, 11978-11982
4 Lacks, S.A., *et al.* 1986. *J. Mol. Biol.* 192, 753-765
5 Espinosa-Urgel, M., and Tormo, A. 1993. *Nucl. Acids Res.* 21, 3667-3670
6 Pérez-Martín, J. *et al.* 1988. *Nucl. Acids Res.* 16, 9113-9126

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Transfer of the broad-host-range plasmids from both Gram-positive and Gram-negative bacteria to Brevibacterium methylicum

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Gram-positive facultative methylotrophic coryneform bacterium Brevibacterium methylicum was efficiently transformed with various plasmids using electroporation of intact cells. In addition to the plasmid vectors pEC71 and pZ6-1 constructed on the basis of cryptic plasmids from coryneform bacteria, broad-host-range plasmids pLS5 (derivative of plasmid pMV158 from Streptococcus agalactiae) and RSF1010 belonging to the incompatibility group IncQ from Gram-negative bacteria were found to be present as autonomous structurally unchanged DNA molecules in B. methylicum transformants. With the exception of pZ6-1, all these plasmids were stably maintained in B. methylicum cells grown under non-selective conditions. When plasmid DNAs isolated from B. methylicum were used the highest efficiency of transformation (10^5 transformants/µg DNA) was achieved.

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CONJUGATIVE AND THERMOSENSITIVE CLONING VECTORS FOR MYCOBACTERIA

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In order to develop a transposon mutagenesis system for Mycobacteria, shuttle plasmids capable of conjugative transfer from *Escherichia coli* to *Mycobacterium smegmatis* were constructed. They contained both a pUC derivative which replicates in *E. coli* and a thermosensitive pAL5000, Mycobacterial replicon. Transfer was dependent on the Inc P-type plasmid RP4's transfer functions integrated into the chromosome and also on the presence of it's recognition site mob in the shuttle vector. These vectors allowed the delivery of Tn*611* at frequencies higher than those detected following electroporation of non-conjugative vectors.

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Transfer capacity of different plasmid species in sterile and non-sterile soil

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A collection of plasmids of different incompatibility groups carrying sat1 and sat2 genes (IncFII, IncI1, IncN, IncW, IncP) on Tn7-like transposons and the sat3 gene (IncQ) was available after years of monitoring the distribution of sat genes in different environments. To study the effect of the localization of sat genes on their distribution via horizontal gene transfer *E. coli* strains with the a.m. plasmid species were tested in sterile and nonsterile silt loam. No background of sat genes in culturable bacteria was detected before inoculation of *E. coli* strains. The potential of *E. coli* strains to survive in sterile and nonsterile soil was shown to be high enough to look for gene transfer events in soil. Parallel to soil inoculation, plate matings were performed on yeast extract agar (YEA), YEA with soil extract, and YEA containing soil particles. The plasmids under question were transferred to *E. coli* CV601 (thr^-, leu^-, thi^-, rif^r). For IncQ mobilization the pTH10 plasmid was used as helper plasmid.

Even though all plasmids were transferred in plate matings without any inhibitory effects due to soil components, plasmid transfer of IncFII and IncI1 in *E. coli* CV601 was not detected in sterile and nonsterile soil which might be due to non-optimal spatial conditions for long flexible pili. Conjugative plasmid transfer of the broad host range plasmids IncW, IncN and IncP to *E. coli* CV601 showed comparable transfer frequencies for IncP of about $3.2x10^{-4}$ for nutrient amended sterile soil and $3.3x10^{-4}$ for nutrient amended nonsterile soil, and about $3.1x10^{-5}$ for nonamended sterile soil (see Figure 5 for plasmid characterization). Transfer frequencies for IncN were of about $3.7x10^{-5}$ for sterile soil and $1.1x10^{-4}$ for nonsterile soil with nutrient amendment, and for IncW of about $9.6x10^{-5}$ for nutrient amended sterile soil and $6.1x10^{-5}$ for nutrient amended nonsterile soil.

50 days after incubation no *E. coli* strains were detected whereas indigenous bacteria, carrying a sat2 gene, could be isolated from nutrient amended nonsterile soil experiment with the IncP plasmid. These strains, identified with the BIOLOG identification system, could be assigned to the genus *Rhizobium* and to the *Flavobacterium* group.

Those plasmids supposed to be P-plasmids were retransferred from the two different Rhizobia species and the Flavobacterium to identical *E. coli* CV601. Identity of the donor plasmids was shown by *Eco*RV restriction pattern and the sensitive titre increase test for the respective phage (ike, PR4).

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SPECIFICITY OF "IN VITRO" ORIGIN RECOGNITION BY THE INITIATOR PROTEIN RepB OF PLASMID pMV158

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The streptococcal promiscuous plasmid pMV158 (5536 bp) is the prototype of a number of small rolling circle-replicating plasmids isolated form gram-positive and -negative bacteria (1,2). In addition to replicate in *Streptococcus pneumoniae*, pMV158 can be established in the mesophilic bacteria *Bacillus subtilis* and in the thermophile *S.thermophilus*. All plasmids of the pMV158 family share homologies at their initiator of replication (Rep) proteins and their plus origins of replication (*dso*). Replication of the pMV158 leading strand is mediated by the plasmid-encoded protein RepB (3,4). RepB is a 24.5 kDa protein which has been purified from an *E.coli* expression system. Hydroxyl radical footprinting and filter binding assays on linear DNA (4) showed that RepB protein binds to a set of three iterons (the *bind* region), which is located 84 bp downstream of the site where RepB introduces a strand- and site-specific nick to initiate replication (the *nic* region; Figure 1, and ref. 5). All plasmids of the pMV158 family have iterons of different lenghts, located downstream of their *nic* region. The distance between the first base of the first iteron to the nick site varies between 91 bp (plasmid pKMK1) and 13 bp (plasmid pA1). Rep proteins of plasmids of the family share homologies, which are more pronounced at their N-termini moiety. Those homologies extend the entire Rep proteins in those plasmids that have similar iterons. The distribution of the blocks of homology suggests that the N-terminal moiety of the Rep protein would be involved in nicking DNA, whereas the C-termini would be involved in binding to the iterons (1).

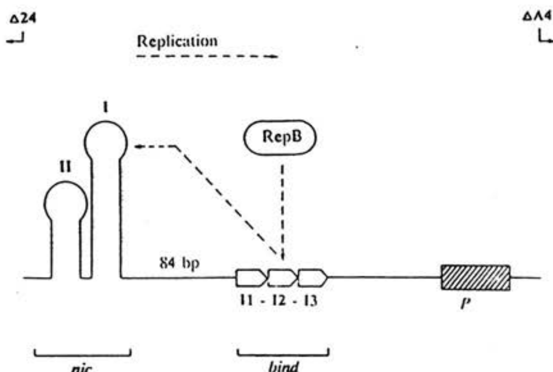

Figure 1. The *dso* of plasmid pLS1. The scheme shows the three iterons where RepB binds (I1-I3), the RepB-nick site on Hairpin I (I), and the putative Hairpin II (II). Position of the $\underline{P}_{cr}$ promoter for transcription of *copG* and *repB* (shadowed box), and direction of replication are also indicated. In the upper part, it is indicated the borders of the deletions so far obtained that approximate most to the *dso* at its left (Δ24) and right (ΔA4) parts.

Initiation of replication of pMV158 is initiated by RepB cleaving the phosphodiester bond GpA within the region 5'-$(G)_7$CTACTACG/AC$(C)_6$-3'. This region is able to generate a secondary structure (Hairpin I), which has been mapped by nuclease S1-treatment of supercoiled DNA. RepB protein is able to relax supercoiled

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DNA of pMV158 and of various of its derivatives at 37°C, although 100% relaxation could not be achieved. However, all molecules were relaxed at 45°C, and even at 60°C. Supercoiled DNAs of the related plasmids pFX2 and pE194 were treated with RepB protein: whereas pFX2 DNA was sensitive to RepB, no relaxation was observed with DNA of pE194.

To determine the specificity of recognition of RepB to its target DNA, synthetic single-stranded (ss) 23-mer oligonucleotides containing base-changes at the *nic* region of pMV158 have been prepared. They have been labelled at their 5'-or 3'-ends, and treated with RepB at various temperatures. The results showed that: i) RepB protein is only able to nick ss-oligonucleotides, but not double-stranded oligonucleotides; ii) secondary structures does not seem to be needed on linear ssDNA; iii) RepB does not remain covalently attached to the 5'-end of the cleaved ssDNA, although a transient bond may exist, and iv) changes at the GpA dinucleotide are allowed if the G residue is changed by a A, but not by a T or C (Table 1). Preliminar results have shown that purified RepB protein is also able to seal the nick but only if previous RepB-DNA contacts are allowed.

Table 1. Specificity of recognition of purified RepB to 23-mer oligonucleotides.

Oligo	Sequence (5'-3')	Nicking
wt	GGGGGGGCTACTACG/ACCCCCCC	+++
C/A	GGGGGGGCTACTACC/ACCCCCCC	-
A/A	GGGGGGGCTACTACA/ACCCCCCC	++
T/A	GGGGGGGCTACTACT/ACCCCCCC	-
G/T	GGGGGGGCTACTACG/TCCCCCCC	++
G/G	GGGGGGGCTACTACG/GCCCCCCC	-
G/C	GGGGGGGCTACTACG/CCCCCCCC	++
X	TGCTTCCGTACTACG/ACCCCCCA	+++
comp	GGGGGGGTCGTAGTAGCCCCCCC	-

References

1. del Solar,G., Moscoso,M. and Espinosa,M. (1993). Mol. Microbiol. **8**:789-796.
2. M.Espinosa,M., G.del Solar, M.Moscoso,M., G.Kramer and P.Acebo. Accompanying Abstract.
3. Lacks,S.A., López,P., Greenberg,B. and Espinosa,M. (1986). J. Mol. Biol. **192**:753-765.
4. de la Campa,A.G., del Solar,G. and Espinosa,M. (1990). J. Mol. Biol. **213**:247-262.
5. del Solar,G., Moscoso,M. and Espinosa,M. (1993). Mol. Gen. Genet. **237**:65-72.

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Sequence analysis and comparison of the replication gene *trfA*, and the Tra2 genes *trbA* and *trbB*, from broad host range IncP plasmids RK2 and R751.

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Broad host range plasmids have gained considerable attention over the past years due to their promiscuous nature. The IncP family is the best and most extensively studied. They have been isolated from a variety of bacterial species in many parts of the world. Although diverse with respect to their different antibiotic resistance markers and restriction maps, southern blotting showed that the plasmids have a similar genetic organization, thus revealing their close relationship. Sequence comparisons revealed highly conserved regions containing some of the essential genes of these plasmids.

The IncP plasmids can propagate themselves by two methods: I, vegetative replication, where the plasmid copies itself, in order for the two daughter cells to recieve a copy of the plasmid before the bacterial division, or II, by conjugational transfer where a donor cell makes contact with a recipient cell, then transfers one of the plasmid strands to the new host, where the plasmid can be established.

Vegetative replication of IncP plasmids has been demonstrated to rely on the expression of a plasmid-encoded replication gene *trfA* (mapping at 16kb-18kb), and a *cis*-acting site *oriV* (mapping at 12.4kb-13kb) (Figurski and Helinski, 1979, Thomas *et al.*, 1980), from where unidirectional replication starts (Meyer and Helinski, 1977). The RK2 *trfA* operon is transcribed counterclockwise (Thomas, 1984) and the polypeptide products have been shown to be SSB, 116aa (an *E.coli* SSB homolog (Thomas and Sherlock, 1990, Jovanovic *et al.*, 1992)), L-TrfA, 382aa and S-TrfA, 285aa (the replication proteins) (Shingler and Thomas, 1984b, Smith and Thomas, 1984). L-TrfA and S-TrfA are translated from the same reading frame with the L-TrfA start codon 294 bp upstream of the S-TrfA start codon. This results in a S-TrfA identical to the 285 amino acid C-terminus of L-TrfA. *oriV* contains nine direct repeats to which TrfA can bind (TrfA boxes) (Stalker and Thomas, 1981,). These boxes are organized in three clusters of one, three and five repeats, with the group of five closest to the replication start point. TrfA binding to these five boxes initiates aggregation of host proteins and the start of replication (Pinkney *et al.*, 1988). It has been shown that expression of S-TrfA is proficient to support replication of an *oriV* plasmid in some bacteria. However, in *Pseudomonas aeruginosa* the expression of L-TrfA was necessary to maintain the *oriV* plasmid (Durland and Helinski, 1987, Shingler and Thomas, 1989). Previously it has been shown that TrfA from RK2 can complement replication from R751 *oriV* and vice versa. This and the fact that the TrfA boxes in *oriV* and their organization is highly conserved between RK2 and R751 (Smith and Thomas, 1985) indicated that the TrfA proteins from these two plasmids would have functional similarities. An analysis of their sequence homology could yield information about conservation of functional domains.

Conjugational transfer of IncP plasmids is dependent on two regions, Tra1 (mapping at 40kb-54kb) and Tra2 (mapping at 18kb-30kb). The Tra1 region has been shown to be involved in the transfer of DNA after the bacterial mating has occurred. *oriT* is part of this region and the gene where proteins bind and nicking occurs before one strand is transfered into the recipient (Guiney *et al.*; 1988, 1989). Mutations and deletion in the Tra2 region showed that these genes are essential for establishing the initial mating pair formation (mpf) between donor and recipient bacteria (Palombo *et al.*, 1989, Lessl *et al.*, 1993). The genes in the Tra2 region have been designated *trb* genes. These are translated from two clockwise transcripts. The first promoter lies upstream of *trbA* gene. The second promoter lies upstream of *trbB*. The *trbB,C,D,E,F,G,H,I,J,K,L* genes are all translated from this polycictronic messenger.

The expression of genes for initiating either vegetative replication or conjugational transfer seems to be controlled by the *korA* gene from the "central control operon", as KorA

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stimulates *trbA* expression and represses *trfA* expression (both on transcriptional level) (Jagura-Burdzy *et al.*, 1992). The switching between the two modes of replication may become clearer as more data emerge about the interactions of these essential genes.

Sequence analysis and comparisons between RK2 and R751 of the *trfA* and *trb* genes and the peptide products may provide a fuller picture of the organization, importance, function and regulation of the genes in these regions, and ideas of where to direct future genetic and molecular studies on the replication and propagation of these plasmids. Here we report the sequence comparison of the region containing the replication gene *trfA*, and the Tra2 genes, *trbA* and *trbB*.

A 7.3 kb *Eco*RI to *Pst*I fragment from R751 (coordinates 14.9kb - 22.2kb) was cloned into pBR322 and sequenced. This fragment covers the *trfA* gene and part of the Tra2 region. The sequence showed that S-TrfA is very highly conserved between the two plasmids on DNA as well as on protein level. However, the region of a proposed DNA binding domain (helix-turn-helix) (Smith and Thomas, 1984) 130 amino acids from the C-terminus, seemed less conserved than adjacent regions. But a closer analysis revealed that the amino acids were semi-conserved in that they all retained charge, polarity, and size, probably creating a similar domain. Sequence further upstream of S-TrfA showed that although the R751 *trfA* gene encoded both a large and a small product the extra N-terminus of L-TrfA is very poorly conserved. As mentioned earlier L-TrfA is essential for effecient RK2 replication in *P.aeruginosa*. The lack of a conserved R751 L-TrfA was therefore surprising, as the N-terminus part may be important in determining the host range of the plasmid. This can be an indication that R751 has a different host range from RK2. This is currently being investigated. The *SSB* region was also highly conserved, but a possible role for SSB has yet to be demonstrated.

There are two operator sites in the RK2 *trfA* promoter, one for KorA and one for KorB. The KorA operator (11bp palindrome) overlaps the -10 promoter sequence and the KorB operator (13bp palindrome) is located just upstream of the -35 sequence (Theophilus, *et al.*, 1985). The sequence and location of these two operator palindromes for KorA and KorB are conserved on R751 suggesting a similar pattern of regulation of this important operon. This has been verified by promoter fusion assays, testing the regulation of the R751 *trfA* promoter by KorA and KorB.

trbA is part of the Tra2 region and encodes TrbA, of 120aa. The RK2 *trbA* promoter is organized as a face to face divergent promoter with the *trfA* promoter (Shingler and Thomas, 1984a, Jagura-Burdzy *et al.* 1992). Regulation of the *trfA* and *trbA* genes is simultaneous. KorA represses transcription of *trfA* and activates the transcription of *trbA*. KorB represses *trfA* and *trbA* transcription (Jagura-Burdzy *et al.*, 1992). Sequence analysis of R751 *trbA* shows an identical organization of the genes and the DNA and protein sequence is also highly conserved. The 3' end of the RK2 *trbA* mRNA has the potential to form a large stem-loop structure (Motallebi-Vershareh, 1992). However, this region is not very well conserved on DNA level of R751 *trbA*, so the exact role of this secondary mRNA structure has not yet been elucidated.

Downstream of RK2 *trbA* is *trbB*, encoding TrbB, of 320aa, and in the intergenic region between *trbA* and *trbB* is a KorB operator, responsible for repressing *trbB* transcription (Smith and Thomas, 1983, Shingler and Thomas, 1984a, Bechhofer *at al.*, 1986). This is also conserved on R751 indicating a similar pattern of regulation, but how KorB regulates transcription 120bp upstream of the *trbB* promoter remains to be established. Activation of *trbA* by KorA may somehow repress *trbB* expression. The R751 sequence revealed a new palindrome situated between the -35 and -10 sequences of the R751 *trbB* promoter indicating a possible involvement in regulation of *trbB* expression unique to R751, which needs to be identified. Sequence identity on DNA level is very low (40%) in the region between *trbA* and *trbB*, apart from the KorB operator and the -10 and -35 regions of the *trbB* promoter, suggesting that the spacing is somehow important for regulation.

Aligning the proteins sequences from RK2 and R751 with minimal number of gaps gave the following similarity/identity: S-TrfA 88%/79%, L-TrfA (N-terminal only) 49%/35%, SSB 82%/72%, TrbA 86%/76% and TrbB 88%/82%. The sequence of the *trfA* gene and part of the Tra2 region revealed that apart from promoter and operator sites, the intergenic regions are less conserved than translated DNA regions.

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References:

Bechhofer,D.H., Kornacki,J.A., Firshein,W. and Figurski,D.H. (1986). Gene control in broad host range plasmid RK2: expression, polypeptide product and multiple regulatory functions of *korB*. Proc.Natl.Acad.Sci.USA, 83:294-398.

Durland,R.H. and Helinski,D.R. (1987). The sequence encoding the 43-kilodalton *trfA* protein is required for efficient replication or maintenance of minimal RK2 replicons in *Pseudomonas aeruginosa*. Plasmid, 18:164-169.

Figurski,D.H. and Helinski,D.R. (1979). Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided *in trans*. Proc.Natl.Acad.Sci.USA, 76:1648-1652.

Guiney,D.G., Deiss,C and Simnad,V. (1988) Location of the relaxation complex nick site within the minimal origin of transfer of RK2. Plasmid. 20:259-265.

Guiney,D.G., Deiss,C., Simnad,V., Yee,L., Pansegrau,W. and Lanka,E. (1989). Mutagenesis of the Tra1 core region of RK2 by using Tn5: identification of plasmid-specific transfer genes. J.Bact., 171:4100-4103.

Jagura-Burdzy,G. Khanim,F. Smith,C.A. and Thomas,C.M. (1992). Crosstalk between plasmid vegetative replication and conjugative transfer: repression of the *trfA* operon by *trbA* of broad host range plasmid RK2. Nucl.Acid Res., 20:3939-3944.

Jovanovic,O.S., Ayres,E.K. and Figurski,D.H. (1992). The replication initiator operon of promiscous plasmid RK2 encodes a gene that complements an *Eschericia coli* mutant defective in single-stranded DNA-binding protein. J.Bact., 174:4842-4846.

Lessl,M., Balzer,D., Weyrauch,K. and Lanka,E. (1993). The mating pair formation system of plasmid RP4 defined by RSF1010 mobilization and donor-specific phage probagation. J.Bact., 175:6415-6425.

Meyer,R. and Helinski,D.R. (1977). Unidirectional replication of the P-group plasmid RK2. Biochim.Biophys.Acta, 478:109-113.

Motallebi-Vershareh,M., Balzer,D., Lanka,E., Jagura-Burdzy,G. and Thomas,C.M. (1992). Conjugative transfer functions of broad-host-range plasmid RK2 coregulated with vegetative replication. Molecular Microbiology, 6:907-920.

Palombo,E.A., Yusoff,K., Stanisich,V.A., Krishnapillai,V. and Willets,N.S. (1989). Cloning and genetic analysis of *tra* cistrons of the Tra2/Tra3 region of plasmid RP1. Plasmid, 22:59-69.

Pinkney,M., Diaz,R., Lanka,E. and Thomas,C.M. (1988) Replication of mini RK2 plasmid in extracts of *Escherichia coli* requires plasmid-encoded protein TrfA and host-encoded proteins DnaA, B, G, DNA Gyrase and DNA Polymerase III. J.Mol.Biol., 203:927-938.

Shingler,V. and Thomas,C.M. (1984a) Transcription in the *trfA* region of broad host range plasmid RK2 is regulated by *trfB* and *korB*. Mol.Gen.Genet., 195:523-529.

Shingler,V. and Thomas,C.M. (1984b) Analysis of the *trfA* region of broad host range plasmid RK2 by transposon mutagenesis and identification of the polypeptide products. J.Mol.Biol., 175:229-250.

Shingler,V. and Thomas,C.M. (1989). Analysis of nonpolar insertion mutations in the *trfA* gene of IncP plasmid RK2 which affect its broad-host-range property. Biochimica et Biophysica Acta, 1007:301-308.

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Smith,C.A. and Thomas,C.M. (1983) Deletion mapping of *kil* and *kor* functions in the *trfA* and *trfB* regions of broad host range plasmid RK2. Mol.Gen.Genet., 190:245-254.

Smith,C.A. and Thomas,C.M. (1984). Nucleotide sequence of the *trfA* gene of broad host-range plasmid RK2. J.Mol.Biol., 175:251-262.

Smith,C.A. and Thomas,C.M (1985). Comparison of the nucleotide sequences of the vegetative replication origins of broad host range IncP plasmids R751 and RK2 reveals conserved features of probable functional importance. Nucl.Acid Res., 13:557-572.

Stalker,D.M., Thomas,C.M. and Helinski,D.R. (1981). Nucleotide sequence of the region of the origin of replication of the broad host range plasmid RK2. Mol.Gen.Genet., 181:8-12.

Theophilus,B.D.M., Cross,M.A., Smith,C.A. and Thomas,C.M. (1985). Regulation of the *trfA* and *trfB* promotors of broad host range plasmid RK2: identification of sequences essential for regulation by *trfB/korA/korD*. Nucl.Acid Res., 13:8129-8142.

Thomas,C.M., Meyer,R. and Helinski,D.R. (1980). Regions of broad-host-range plasmid RK2 which are essential for replication and maintenance. J.Bact., 141:213-222.

Thomas,C.M. (1984). Genetic evidence for the direction of transcription of the *trfA* gene of broad host range plasmid RK2. J.G.M., 130:1641-1650.

Thomas,C.M. and Sherlock,S. (1990). Broad host range plasmid RK2 encodes a polypeptide related to single-stranded DNA binding protein (SSB) of *Escherichia coli*. Nucl.Acid Res., 18:2812.

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SELECTION OF MUTANTS WHICH IMPROVE PLASMID R1 REPLICATION PROMOTED BY THE DEREPRESSION OF THE PARD STABILITY SYSTEM

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parD is an killer stability system of the antibiotic resistance factor R1 that is organized as an operon of two genes denominated *kis* and *kid* (1,2). These two genes code for two proteins of 9.3 and 12 kDa respectively. The Kid protein is a citytoxin and the Kis protein is an antagonist of Kis (3). The system is autoregulated by the coordinate action of the Kis and Kid proteins (2) but it can be derepressed by a mutation in the *repA* gene that reduces the efficiency of plasmid replication. Derepression of the wild-type *parD* system by the mutation in *repA* stabilizes efficiently the plasmid and is associated with counterselection of plasmid-free segregants and also with a delay in the growth of plasmid containing cells. Deletion of *copB* gene in the plasmid carrying the *repA* mutation increases copy number, results in transcriptional repression of the *parD* system and prevents the interference with cell growth observed in the derepressed situation (Ruiz-Echevarría *et al,* submitted).
We now report that expontaneous mutations that restore the growth rate of cells containing the *parD*-derepressed *repA*-mutant, contain mutations that improve the efficiency of plasmid replication replication. Two type of genetic changes are found in each mutant: one of them is either a point mutation, an insertion or a deletion that inactivates *kid*, the killer component of *parD,* and the other is a true reversion of the original amber mutation in *repA*. These results underline complex relations between *parD* expression and plasmid replication and indicate that the *parD* system can also lead, in plasmid replication mutants, to the selection of mutations that improve the efficiency of plasmid replication.

(1) Bravo,A., de Torrontegui,G., and Díaz,R. Identification of components of a new stability system, *parD*, of plasmid R1 that is close to its origin of replication. Mol. Gen. Genetics 210, 101-110 (1987)
(2). Ruiz-Echevarría, M.J., Berzal-Herranz,A., Gerdes,K. and Díaz-Orejas,R. The *kis* and *kid* genes of *parD* maintenance system of plasmid R1 form an operon that is autoregulated at the level of transcription by the coordinate action of the Kis and Kid proteins. Mol. Microbiol. 5, 2685-2693 (1991)
(3) Bravo,A., Ortega,S., de Torrontegui,G., and Díaz,R. Killing of *Escherichia coli* cells modulated by components of the stability system *parD* of plasmid R1. Mol. Gen. Genetics 215, 146-151 (1988)

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SUMMARY

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Summary

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 important ecological questions while for the ecologists it was important 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. Inevitably this account will be biased towards the perspective of a molecular biologist but I hope that it is seen as valid and useful for both groups. The topics will be 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 (Chattoraj) and broad host range IncP plasmid RK2 (Helinski) which were described, illustrated how replication origins which are activated by binding of a Rep protein to repeated sequences (iterons) in the origin are dependent on efficient interaction which host intracellular environment including host-encoded replication proteins such as DnaA, so that factors determining this interaction may be critical in determining which host a plasmid can replicate in. The C-terminus of the replication initiator protein TrfA is highly conserved among members of the IncP family (Thorsted) 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 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 *E. coli* can be overcome by reducing the temperature to 25°C or by a point mutation in the pPS10 Rep protein (Fernandez-Tresguerres). This observation fits with the point emphasised by John Fry, 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 Gram negative bacterial species (Espinosa); and the theta replication family of pSM19035 which has a broad host range within Gram-positive (Alonso). Studies with the pMV158

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derivative pLS1 are uncovering the way that plasmid gene expression signals respond to stationery phase (Espinosa Jnr). This may provide a first step in opening up the area of plasmid survival in nongrowing 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 in order 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 (Wilmotte). 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 IncPα and β subgroups was recognised while PCR probes for coding regions of essential problems were described by Van Elsas and Thomas. The latter were based on 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 many depths of lake sediments (Pickup), 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. IncPβ probes suggest that these may be related to the 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 (de la Cruz, Lanka) was extended to *Rhizobium leguminosarium* in which the first new plasmid replicon to be isolated has sequence similar to Ti and Ri plasmids (Turner) as well as to IncP plasmids in the *inC 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 (Nordstrom) in order to appreciate how variations in plasmid copy number per cell, multimerization and the differences in growth rate of plasmid

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positive and negative can influence the apparent rate of plasmid loss. Mechanisms which ensure at least random segregation by resolution of mulitmers 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 (*ccd* - Couturier) or antisense repressor RNA (*sok* - Gerdes). Of particular interest were the *parD/E* genes of RK2 (Helinski) and *parD* system of R1 (Diaz-Orejas) 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 still are poorly understood despite recent progress (Jensen) and the possibility of building a bridge to physiological conditions and external factors is still distant.

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 apparatuses of many other conjugative plasmids like the IncW group (de la Cruz) as well as having many similarities to the *vir* system of Ti plasmids. 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 (Jagura-Burdzy) hint at control of transfer in response to copy number, physiology or growth phase although 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 and why is there 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 depression event followed by 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 (Smalla). The role of pili is still not understood fully and indeed bacteria-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 right through to integration of the transferred DNA once it has entered the recipient's nucleus (Hohn). For interbacterial matings restriction imposes a major barrier which can be overcome in a number of ways: saturation of the restriction system by multiple rounds of transfer and delivery of an antirestrictive gene whose product blocks the action of type I restriction endonucleases (Wilkins). 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

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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 (Wellington, Molin, Palomares) 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 a unit 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 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. Analysing the response of 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. First, 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 *P. aeruginosa* virulence in a mouse model system, providing a possibly 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 (Thomas). Second, 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 (Baquero) and the selective advantage of each step can be dissected by site directed mutagenesis. Plasmids may also promote the combination of single changes at higher rate than double mutation but this has not been investigated. Some plasmids actually encode UV-repair, recombination and integrate type

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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 (Battaglia). 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* (Courvalin).

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 it will die when its job is done - for example in the degradation of an environmental pollutant, or in the case of a plasmid its transfer to a host can be inhibited by it 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 Colici E1 gene and the immunity gene which regulates it (Diaz). 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 cross talk between different regulatory systems (de Lorenzo) 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

Conclusions

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 biologist 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.

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List of Invited Speakers

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Workshop on

MOLECULAR BIOLOGY AND ECOLOGY OF GENE TRANSFER AND PROPAGATION PROMOTED BY PLASMIDS

List of Invited Speakers

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Workshop on

MOLECULAR BIOLOGY AND ECOLOGY OF GENE TRANSFER AND PROPAGATION PROMOTED BY PLASMIDS

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247 **Workshop on Pathogenesis-related Proteins in Plants.**
Organized by V. Conejero and L. C. Van Loon. Lectures by L. C. Van Loon, R. Fraser, J. F. Antoniw, M. Legrand, Y. Ohashi, F. Meins, T. Boller, V. Conejero, C. A. Ryan, D. F. Klessig, J. F. Bol, A. Leyva and F. García-Olmedo.

248 Beato, M.:
Course on DNA - Protein Interaction.

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251 **Lecture Course on Approaches to Plant Development.**
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253 **Workshop on Genome Expression and Pathogenesis of Plant RNA Viruses.**
Organized by F. García-Arenal and P. Palukaitis. Lectures by D. Baulcome, R. N. Beachy, G. Boccardo, J. Bol, G. Bruening, J. Burgyan, J. R. Díaz Ruiz, W. G. Dougherty, F. García-Arenal, W. L. Gerlach, A. L. Haenni, E. M. J. Jaspars, D. L. Nuss, P. Palukaitis, Y. Watanabe and M. Zaitlin.

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Organized by H. Saedler, J. P. Beltrán and J. Paz Ares. Lectures by P. Albersheim, J. P. Beltrán, E. Coen, G. W. Haughn, J. Leemans, E. Lifschitz, C. Martin, J. M. Martínez-Zapater, E. M. Meyerowitz, J. Paz-Ares, H. Saedler, C. P. Scutt, H. Sommer, R. D. Thompson and K. Tran Thahn Van.

259 **Workshop on Transcription and Replication of Negative Strand RNA Viruses.**
Organized by D. Kolakofsky and J. Ortín. Lectures by A. K. Banerjee, M. A. Billeter, P. Collins, M. T. Franze-Fernández, A. J. Hay, A. Ishihama, D. Kolakofsky, R. M. Krug, J. A. Melero, S. A. Moyer, J. Ortín, P. Palese, R. G. Paterson, A. Portela, M. Schubert, D. F. Summers, N. Tordo and G. W. Wertz.

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265 **Workshop on Adhesion Receptors in the Immune System.**
Organized by T. A. Springer and F. Sánchez-Madrid. Lectures by S. J. Burakoff, A. L. Corbi-López, C. Figdor, B. Furie, J. C. Gutiérrez-Ramos, A. Hamann, N. Hogg, L. Lasky, R. R. Lobb, J. A. López de Castro, B. Malissen, P. Moingeon, K. Okumura, J. C. Paulson, F. Sánchez-Madrid, S. Shaw, T. A. Springer, T. F. Tedder and A. F. Williams.

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267 **Workshop on Role of Glycosyl-Phosphatidylinositol in Cell Signalling.**
Organized by J. M. Mato and J. Larner. Lectures by M. V. Chao, R. V. Farese, J. E. Felíu, G. N. Gaulton, H. U. Häring, C. Jacquemin, J. Larner, M. G. Low, M. Martín Lomas, J. M. Mato, E. Rodríguez-Boulan, G. Romero, G. Rougon, A. R. Saltiel, P. Strålfors and I. Varela-Nieto.

268 **Workshop on Salt Tolerance in Microorganisms and Plants: Physiological and Molecular Aspects.**
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Toro. Lectures by L. Adler, E. Blumwald, V. Conejero, W. Epstein, R. F. Gaber, P. M. Hasegawa, C. F. Higgins, C. J. Lamb, A. Läuchli, U. Lüttge, E. Padan, M. Pagès, U. Pick, J. A. Pintor-Toro, R. S. Quatrano, L. Reinhold, A. Rodríguez-Navarro, R. Serrano and R. G. Wyn Jones.

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5 **Workshop on Structure of the Major Histocompatibility complex.**
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6 **Workshop on Behavioural Mechanisms in Evolutionary Perspective.**
Organized by P. Bateson and M. Gomendio. Lectures by J. R. Alberts, G. W. Barlow, P. Bateson, T. R. Birkhead, J. Carranza, C. ten Cate, F. Colmenares, N. B. Davies, R. I. M. Dunbar, J. A. Endler, M. Gomendio, T. Guilford, F. Huntingford, A. Kacelnik, J. Krebs, J. Maynard Smith, A. P. Møller, J. Moreno, G. A. Parker, T. Redondo, D. I. Rubenstein, M. J. Ryan, F. Trillmich and J. C. Wingfield.

7 **Workshop on Transcription Initiation in Prokaryotes.**
Organized by M. Salas and L. B. Rothman-Denes. Lectures by S. Adhya, H. Bujard, S. Busby, M. J. Chamberlin, R. H. Ebright, M. Espinosa, E. P. Geiduschek, R. L. Gourse, C. A. Gross, S. Kustu, J. Roberts, L. B. Rothman-Denes, M. Salas, R. Schleif, P. Stragier and W. C. Suh.

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8 **Workshop on the Diversity of the Immunoglobulin Superfamily.**
Organized by A. N. Barclay and J. Vives. Lectures by A. N. Barclay, H. G. Boman, I. D. Campbell, C. Chothia, F. Díaz de Espada, I. Faye, L. García Alonso, P. R. Kolatkar, B. Malissen, C. Milstein, R. Paolini, P. Parham, R. J. Poljak, J. V. Ravetch, J. Salzer, N. E. Simister, J. Trinick, J. Vives, B. Westermark and W. Zimmermann.

9 **Workshop on Control of Gene Expression in Yeast.**
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10 **Workshop on Engineering Plants Against Pests and Pathogens.**
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11 **Lecture Course on Conservation and Use of Genetic Resources.**
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12 **Workshop on Reverse Genetics of Negative Stranded RNA Viruses.**
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13 **Workshop on Approaches to Plant Hormone Action.**
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14 **Workshop on Frontiers of Alzheimer Disease.**
Organized by B. Frangione and J. Ávila. Lectures by J. Ávila, K. Beyreuther, D. D. Cunningham, A. Delacourte, B. Frangione, C. Gajdusek, M. Goedert, Y. Ihara, K. Iqbal, K. S. Kosik, C. Milstein, D. L. Price, A. Probst, N. K. Robakis, A. D. Roses, S. S. Sisodia, C. J. Smith, W. G. Turnell and H. M. Wisniewski.

15 **Workshop on Signal Transduction by Growth Factor Receptors with Tyrosine Kinase Activity.**
Organized by J. M. Mato and A. Ullrich. Lectures by M. Barbacid, A. Bernstein, J. B. Bolen, J. Cooper, J. E. Dixon, H. U. Häring, C.- H. Heldin, H. R. Horvitz, T. Hunter, J. Martín-Pérez, D. Martín-Zanca, J. M. Mato, T. Pawson, A. Rodríguez-Tébar, J. Schlessinger, S. I. Taylor, A. Ullrich, M. D. Waterfield and M. F. White.

16 **Workshop on Intra- and Extra-Cellular Signalling in Hematopoiesis.**
Organized by E. Donnall Thomas and A. Grañena. Lectures by M. A. Brach, D. Cantrell, L. Coulombel, E. Donnall Thomas, M. Hernández-Bronchud, T. Hirano, L. H. Hoefsloot, N. N. Iscove, P. M. Lansdorp, J. J. Nemunaitis, N. A. Nicola, R. J. O'Reilly, D. Orlic, L. S. Park, R. M. Perlmutter, P. J. Quesenberry, R. D. Schreiber, J. W. Singer and B. Torok-Storb.

17 **Workshop on Cell Recognition During Neuronal Development.**
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18 **Workshop on Molecular Mechanisms of Macrophage Activation.**
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19 Workshop on Viral Evasion of Host Defense Mechanisms.
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20 Workshop on Genomic Fingerprinting.
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21 Workshop on DNA-Drug Interactions.
Organized by K. R. Fox and J. Portugal. Lectures by J. B. Chaires, W. A. Denny, R. E. Dickerson, K. R. Fox, F. Gago, T. Garestier, I. H. Goldberg, T. R. Krugh, J. W. Lown, L. A. Marky, S. Neidle, D. J. Patel, J. Portugal, A. Rich, L. P. G. Wakelin, M. J. Waring and C. Zimmer.

22 Workshop on Molecular Bases of Ion Channel Function.
Organized by R. W. Aldrich and J. López-Barneo. Lectures by R. W. Aldrich, C. M. Armstrong, P. Ascher, K. G. Beam, F. Bezanilla, S. C. Cannon, A. Castellano, D. Clapham, A. Ferrús, T. Hoshi, A. Konnerth, R. Latorre, J. Lerma, J. López-Barneo, R. MacKinnon, G. Mandel, A. Marty, C. Miller, B. Sakmann, B. Soria, W. Stühmer and W. N. Zagotta.

Instituto Juan March (Madrid)

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