Instituto Juan March
de Estudios e Investigaciones

52 CENTRO DE REUNIONES INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Enzymology of DNA-Strand Transfer Mechanisms

Organized by

E. Lanka and F. de la Cruz

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INTRODUCTION

Fernando de la Cruz and Erich Lanka

This year we celebrate the 50th anniversary of the discovery of bacterial conjugation by Lederberg and Tatum. Since then, conjugation has been a hot matter of active research at various levels: basic advance of knowledge, as a technological tool, by its implication in bacterial ecology (including medically important issues) and evolution, etc.

From a general point of view, we now know it involves the assembly and operation of a complicated structure that can be compared with the small ribosomal subunit, at least in the number of components. Conjugation allows the passage of a single-stranded DNA (that can be as large as the entire bacterial chromosome - a string of 5 million nucleotides) across four bacterial membranes. Amazingly, the process can be of very broad host range, with several known examples of trans-kingdom conjugation. By far the most conspicuous is T-DNA transfer from Agrobacterium tumefaciens to plant cells, a process considered to be a specialized form of bacterial conjugation.

Plasmid conjugation is of actuality also because of the use of exogenous DNA in release experiments, an ecological issue that was discussed at a related workshop in this Institute (Thomas et al., 1994).

The molecular complexity of bacterial conjugation allocates it to the intersection of apparently unrelated research topics, from rolling-circle replication and DNA-strand transfer mechanisms to macromolecular transport through biological membranes. We reasoned that first front research in bacterial conjugation should not limit itself to the knowledge produced by inbreeding. We considered it will most profit from the input of leading experts in the above mentioned flanking areas, by adopting an interdisciplinary approach. Thus, we organized the workshop with strong emphasis in the awareness that the contribution of the flanking areas can trigger in our field. As a nice by-product, the achievements of bacterial conjugation research will be better appreciated by a broader audience.

Hopefully, all participants will benefit from provoking questions and ideas launched across the different fields. With this idea in mind, the keynote lecture was chosen to alert the audience on the crucial importance of the knowledge of the 3D structures of proteins to investigate biochemical processes using topoisomerases as a model system.

The first section of the meeting focused on rolling-circle replication systems, which resemble the DNA processing reactions during conjugation. These were compared at a later

section to other DNA-strand transferases, such as transposases and resolvases. The core sections included the topics of conjugation and T-DNA transfer. The utilization by bacterial conjugation of a complex DNA transport machinery bears analogies with other processes of macromolecular transport through membranes, which constituted another section of the workshop.

In our opinion, the meeting succeeded in presenting the facts of conjugation in a way that could be discussed as pertaining to the selected fields. Perhaps one of the most difficult things was to find a suitable and inclusive title for our enterprise. Our initial idea ("Sources of inspiration for bacterial conjugation") found its way as the headline for the final Round Table discussion which involved many of the participants in a lively and "inspiring" discussion. Although gene transmission by bacterial conjugation is being studied now for 50 years, it is only since very recently that questions dealing with the mechanistic principle were asked and could be answered at the molecular level of enzymes. Consequently, "Enzymology of DNA-strand transfer mechanisms" was chosen as the most appropriate title to describe the aims of the workshop.

OPENING LECTURE

James C. Wang

CATALYSIS OF DNA BREAKAGE AND REJOINING BY DNA TOPOISOMERASES

James C. Wang
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The DNA topoisomerases can be grouped into three subfamilies: the type IA subfamily represented by bacterial DNA topoisomerase I and eukaryotic DNA topoisomerase III, the type IB subfamily represented by eukaryotic DNA topoisomerase I and the poxvirus topoisomerases, and the type II subfamily. All topoisomerases catalyze DNA breakage and rejoining through the formation of covalent intermediates in which DNA strands are linked to the enzymes *via* phosphotyrosine links.

With the determination of the three-dimensional structures of several fragments of various topoisomerases, molecular pictures for the topoisomerase-catalyzed reactions are beginning to emerge. For the type II enzymes, how ATP binding and hydrolysis are coupled to the maneuver of DNA strands poses challenging mechanistic questions. Some of the biochemical, crystallographic, and mutagenesis results will be summarized.

RCR phage and plasmid replication

Chairperson: Tania A. Baker

INITIATION OF BACTERIOPHAGE P2 ROLLING-CIRCLE DNA REPLICATION Elisabeth Haggård-Ljungquist, Yuanfang Liu and Santanu Dasgupta, Dept. of Genetics, Stockholm University, S-106 91 Stockholm, Sweden

The A protein of bacteriophage P2 initiate DNA replication by a single stranded cut at the origin (*ori*), and the DNA replication proceeds unidirectionally by a modified rolling circle type of replication (1, 2, 3). The P2 A protein belongs a family of proteins involved in the initiation of rolling circle DNA replication [Superfamily I, (4)], and the prototype for this family is the well characterized A protein of phage ØX174. One of the common motifs of this family contains two conserved tyrosine residues. In the case of the ØX174 A protein, the two tyrosine residues have been shown to be able to alternate in catalyzing the cleavage as well as joining reactions (5).

The partially purified P2 A protein very efficiently cleaves single-stranded oligonucleotides containing the *ori* region in vitro, while the ligation reaction is rather inefficient under the conditions used. By in vitro mutagensis we have shown that only one of the conserved tyrosine residues is involved in the cleavage and joining reactions. The tyrosine dispensable for cleavage and ligation is, however, essential at some other stage of the P2 growth cycle, since viable recombinants containing this mutation could not be obtained. The sequence requirements for the cleavage reaction was analysed with a set of oligonucleotides having single base transitions in the nick region, and the results indicate that only five nucleotides are required for efficient cleavage (6).

Since the A protein is unable to recognize and bind to the double-stranded *ori* region (3), we are presently using affinity columns with the P2 A protein to identify host factors that might participate in the complex assembled to initiate the rolling circle replication.

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SITE-SPECIFIC DNA STRAND TRANSFERASE ACTIVITY OF THE INITIATOR OF REPLICATION RepB PROTEIN OF PLASMID pMV158

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Rolling circle replication of the streptococcal plasmid pMV158 (5536 bp) is initiated by the product of the plasmid *repB* gene acting on the plasmid double strand origin (*dso*). The first stage of initiation is the binding of RepB to three 11-bp directly repeated sequences (the *bind* region) which are located 84 bp downstream of the nick site (located within the *nic* region; refs. 1-3). The pMV158-*nic* region has the consensus sequence: 5'-TACTACGAC-3', the protein introducing the nick by a nucleophilic attack on the phosphodiester bond of the dinucleotide GpA. Purified RepB protein relaxes supercoiled DNA from either pMV158 and pMV158-derivatives, yielding open circular and covalently closed relaxed forms. RepB-mediated reactions depend upon protein concentration and temperature (4, 5). *In vitro* RepB protein does not need to bind to the plasmid *bind* region to relax supercoiled DNA (with only the pMV158-*nic* region). Site-directed mutagenesis indicate that the *bind* region is essential for pMV158 replication *in vivo*

RepB protein is able to specifically cleave single-stranded oligonucleotides containing the RepB-nick sequence (4). RepB was also able to join two single-stranded oligonucleotides but only if: a) the oligonucleotide corresponding to the right moiety of the nick sequence (5'-$_{p}$AC...-3') was previously generated by the protein, and b) the left moiety of the nick has the wild type sequence (5'-TACTACG$_{OH}$-3'). Thus, RepB has site-specific DNA strand transferase activity.

The *nic* region is conserved among plasmids of the pMV158 family, which is presently constituted by eleven replicons (1, 5, 6). We have shown that RepB protein recognizes the nick sequences of various plasmids of the pMV158-family (5). This was achieved by the use of either supercoiled DNA or single-stranded oligonucleotides (5). In the former case, RepB-mediated DNA relaxation was achieved when an appropriate DNA supercoiling density was introduced in the target DNA (5). RepB is unable to cleave linear double stranded DNA containing the *nic* region. By site-directed mutagenesis and purification of the mutant proteins, we have been able to identify the tyrosyl residue involved in the strand-transferase activity of RepB.

Several attempts aimed at the trapping of covalent RepB-DNA complexes have failed so far. Through filter-binding assays, we have found that RepB-DNA complexes are sensitive to SDS-treatment, suggesting a non-covalent protein-DNA linkage. We have performed a series of experiments aimed at demonstrating the

nature of the RepB nucleotidyl-transfer reaction. To this end, two diastereoisomers of a single stranded thio-oligonucleotide (forms Sp and Rp; containing the *nic* region) were synthesized and purified. The thio-derivative oligonucleotides were treated with RepB in the presence of a 3'-end labelled oligonucleotide containing the 5'-moiety of the nick sequence ($_{p}TACTAC^{32}pG_{OH}$). With this strategy, the RepB-reaction products consisted of an oligonucleotide containing an internal label and an asymmetric phosphorothioate group. This substrate was purified and treated with specific nucleases, allowing us to determine the chirality of the phosphate involved in the nicking-closing reaction. The results suggest that the nucleophilic attack of RepB on its target DNA is exerted through a transient covalent intermediate. In the case of the staphylococcal plasmid pT181, replication of the leading strand terminates in a RepC-mediated reaction which yields an inactive form of the protein containing a short oligonucleotide (7). This RepC::RepC-oligonucleotide heterodimer (8), also seems to be generated in plasmid pUB110 (9). We have not searched for the existence of these complexes with RepB protein. The existence of a covalent RepB-DNA intermediate could, perhaps, mechanistically explain termination of pMV158 replication by a similar process. Alternatively, termination of replication of pMV158 could follow the pathway proposed for plasmid pC194 (10).

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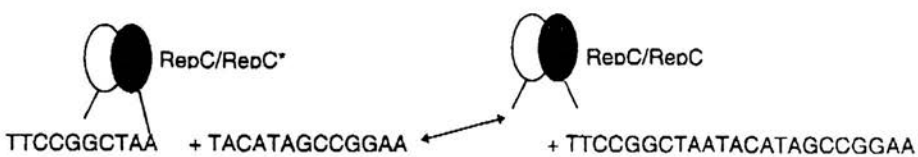
Strand transfer and other properties of RepC, the pT181 initiation protein. **R.P. Novick, R. Jin, and A. Rasooly. Skirball Institute, New York University Medical School, NY, NY, 10016, USA.**

Previous studies have shown that RepC, the initiator of pT181 rolling circle (RC) replication, which is a dimer, is derivatized during replication by the addition of an oligonucleotide to the active site tyrosine of one of its two subunits. The addition of this oligonucleotide, which corresponds to the nucleotides immediately 3' to the initiation nick site, eliminates the nicking, relaxing, and replication activities of the protein, even though only one of the two subunits is modified.

In studies to be reported here, we have compared the native (RepC/RepC) and modified (RepC/RepC*) proteins with respect to the following properties: binding to the leading- or double-strand origin (DSO), DNase I footprinting on supercoiled DNA, and stimulation of cruciform extrusion at the DSO. We show also that RepC/RepC* inhibits the replication-related activities of RepC, including cruciform extrusion, nicking and relaxation, and replication, but that it catalyzes strand transfer.

RepC/RepC* binds to the DSO about half as strongly as RepC/RepC, but causes a mobility shift of only about one half the magnitude of that caused by the native protein. This is because it induces considerably less bending at the DSO than does RepC/RepC. The footprints of the modified and native proteins on supercoiled DNA differ considerably, and the modified form fails to induce cruciform extrusion.

We have used strand transfer to show that the attached oligonucleotide is solely responsible for the lack of activity of RepC/RepC*: the inactive protein can (reversibly) transfer its attached oligonucleotide to a second oligonucleotide consisting of the 5' half of the DSO, thus regenerating fully active RepC/RepC:



Interestingly, this strand transfer reaction is inhibited by oligonucleotides complementary to the cruciform stem, suggesting that secondary structure may be important for the reaction. Additionally, it does not occur with a double-stranded substrate.

The ability of RepC/RepC* to participate in strand transfer suggests a possible mechanism for the termination of pT181 replication: the free subunit carries out a strand transfer between the two copies of the leading strand DSO, circularizing the nascent leading strand and attaching the short extra oligonucleotide to the old displaced leading strand. This junction is now attacked by the attached subunit, which carries out a second strand transfer, recircularizing the displaced leading strand and attaching the short extra oligonucleotide to the protein, generating RepC/RepC* (see Fig. 1).

At this stage, two major unanswered questions are (i) how does the oligonucleotide adduct so profoundly affect the activities of the protein, given that one of the two subunits is unmodified, (ii) how is the DSO configured to permit the strand transfer reactions that are involved in the termination of replication?

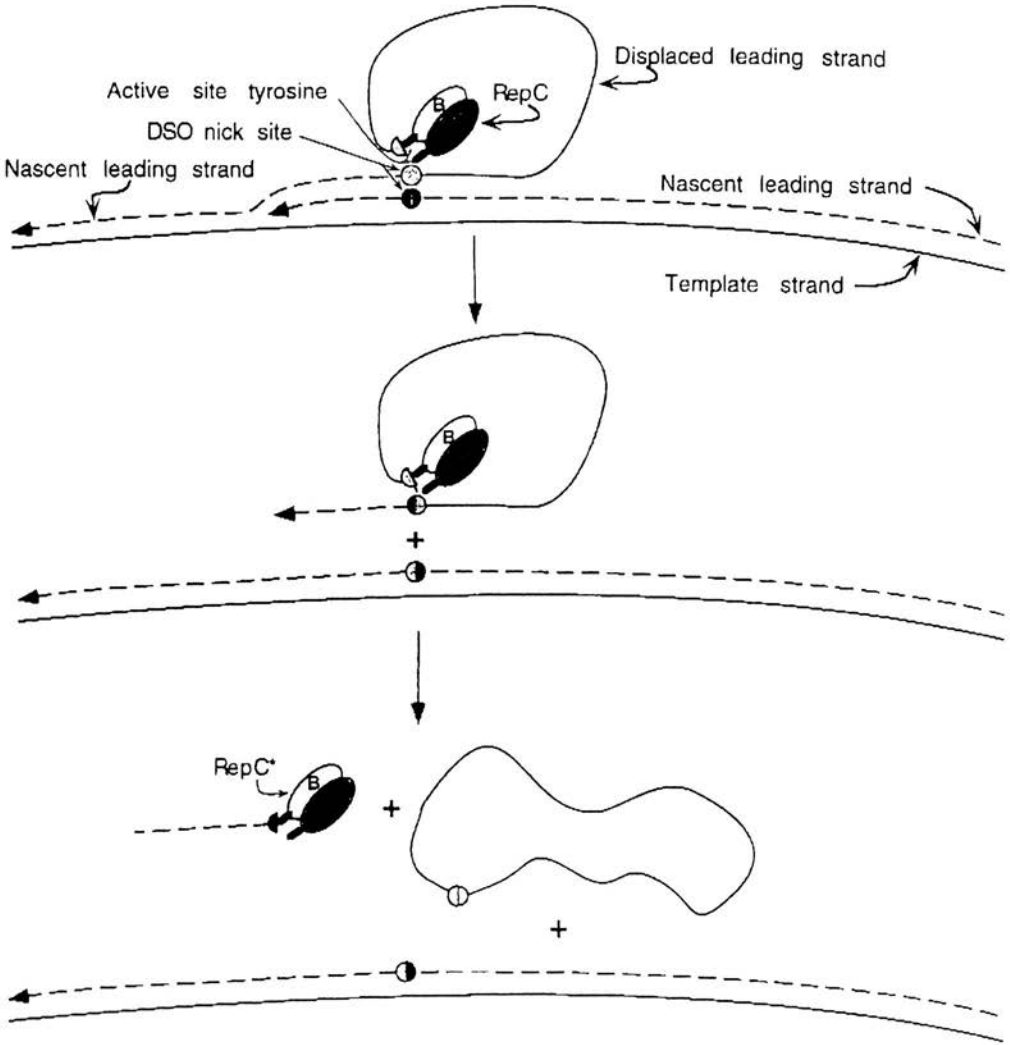


Fig. 1. Possible termination mechanism. Nascent leading strand is extended a shor distance past the DSO nick site, which becomes unpaired from the template. This allows RepC subunit A to perform a strand transfer, switching the two leading strand segments and thus circularizing the new leading strand and transferring the short leading strand e tension to the 3' end of the old leading strand. The attached RepC subunit (B) then catalyzes a second strand transfer, circularizing the displaced leading strand and leaving the short leading strand extension attached to the protein (RepC*), which is then released.

The copy number control of the rolling circle replicating plasmid pUB110 occurs at three levels

J. C. Alonso[1,2], A.-K. Müller[2] and F. Rojo[1]

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Plasmids control their copy number by limiting the amount of the initiator for DNA replication. Plasmid pUB110, that replicates via a rolling circle mechanism like the single-stranded *Escherichia coli* bacteriophages, carries genetic information for the protein that initiates and terminates DNA replication (RepU) and for its own replication control (IncA). Initiation is the rate-limiting step in replication and it is controlled by regulating the availability of the RepU protein at three levels. The expression of the *rep*U gene is controlled by two antisense RNAs (IncA) that interfere with *rep*U mRNA translation (first level of control). The second level of control is by avoiding the re-utilization of the rate-limiting initiator-terminator protein (Rasooly & Novick, 1993. *Science*, **262**, 1048-1050; Rasooly et al., 1994, *EMBO J.*, **13**, 5245-5251). Plasmids of the pUB110 and pT181 families, and perhaps all the rolling circle replicating plasmids, use the strategy of inactivating the initiator-terminator protein (RepC in pT181) during the replication process by the addition of an oligodeoxynucleotide, giving rise to a new form termed RepC*. The active (RepC) and the inactive (RepC*) proteins, which are present in similar relative amounts in the cell, form an inactive RepC-RepC* heterodimer in solution (Rasooly et al., 1994, ibid.). Genetic evidence suggests that RepU protein levels may be regulated by additional uncharacterized mechanisms. RepU overproduction was performed in cells containing the plasmid leading strand replication origin (*dso*), to allow for the accumulation of the RepU*. Polypeptides with apparent molecular masses of 42 (RepU*) and 39 (RepU) kDa were purified, both having the N-terminal sequence expected from the *rep*U gene. The RepU and the RepU-RepU* protein mixture bound specifically to *dso*. At low protein concentrations, about 2 RepU or 6 RepU-RepU* protomers bound to the *dso* region. At higher concentrations, the stoichiometry of the RepU complex remain unaltered, whereas an extended RepU-RepU*-*dso* complex was formed. The promoter for the *rep*U gene was localized downstream of the *dso* region and shown to be represed by the RepU-RepU* complex. The results suggest that the extended RepU-RepU*-*dso* DNA complex interferes with *rep*U promoter utilization. This provides an additional third level of copy number control by limiting transcription of the*rep*U gene.

REPLICATION PROTEIN OF A ROLLING-CIRCLE PLASMID pC194

Noirot-Gros M.F., and S.D. Ehrlich. Génétique microbienne, Institut National de la Recherche Agronomique, Domaine de Vilvert, 78352 Jouy en Josas Cedex, France.

Many plasmids from Gram positive bacteria replicate by a rolling circle (RC) mechanism (1). Their replication is initiated by a protein which introduces a strand-specific nick in the replication origin, designated double stranded origin (DSO), and thus generates a 3'-OH end that serves as a primer for DNA synthesis. Replication of one strand proceeds concomitantly with the displacement of the other. It is terminated by cleavage at the newly synthesized origin and joining the ends, resulting in a circular single-stranded molecule. This molecule is converted to a double-stranded form by replication initiated at the single stranded origin (SSO), which generally involves synthesis of a primer by RNA polymerase.

Several RC replication proteins initiate replication by forming a covalent linkage with the DSO, which involves in all cases a 5'-phosphotyrosyl bond. However, termination of replication is not the same with all the proteins. In the case of phage ΦX174 gene A protein, which is probably the most thoroughly studied, termination of one replication cycle is coupled to initiation of the next. The process occurs by two successive transesterification reactions that involve another tyrosine, present on the same face of a putative local α-helix structure of the protein as the tyrosine linked to DNA (2). This free tyrosine undergoes a transesterification reaction with the newly synthesized origin, forming a novel phosphotyrosyl bond and a 3'-hydroxyl DNA end. The hydroxyl end acts as a nucleophile in a reaction that resolves the 5'-phosphate linkage involving the other tyrosine and forms a covalently closed DNA. The two catalytic tyrosines are thus alternatively engaged in a covalent link with the origin during the successive replication cycles. Several other proteins (DNA topoisomerases, the FLP site-specific recombinase, the γδ resolvase, 3-6), carry out DNA strand transfer by two successive trans-esterification reactions.

In contrast to ΦX174, termination of replication catalyzed by the RepA protein of the plasmid pC194 is not coupled to re-initiation (7). The active sites of pC194 and ΦX174 replication proteins are homologous, but only one of the two catalytic tyrosines is present in RepA, while the other is replaced by a glutamate. It was proposed that the conserved tyrosine initiates replication by forming a covalent link with the origin, and that the glutamate is involved in the termination of replication, by directing the hydrolysis of the newly synthesized origin (8). A transesterification reaction between the 3'-hydroxyl end resulting from the hydrolysis and the 5'-phosphotyrosyl bond completes the replication cycle. The protein is thus released from its substrate and cannot re-initiate replication. The pC194 RepA protein appears to be rather unusual, since it is the first enzyme that catalyses cleavage and joining during DNA strand transfer by a hybrid mechanism, which involves hydrolysis in the resolution of a covalent intermediate formed by transesterification.

From the comparison of the ΦX174 and pC194 replication proteins, it appears that the chemical reaction (transesterification or hydrolysis) which mediates DNA cleavage at the termination step is determined primarily by the functional group (tyrosine or glutamate) present at a key position in the protein active site. To test this hypothesis we generated a RepA mutant derivative in which the glutamate was replaced by a tyrosine. The mutant protein had a normal initiation activity but its termination activity was somewhat impaired.

However, normal initiation and termination was restored by an additional mutation changing a serine, which is not involved in catalysis, to alanine, suggesting a more suitable conformation of this double mutant active site.

To determine whether the substitution of glutamate with tyrosine can promote change of the mechanism from hydrolysis to transesterification, we have tested the RepA mutants for re-initiation activity. The double mutant was capable of re-initiation with a significant frequency (>10 % of termination events). This implies that the novel tyrosine can be transferred to the 5'P DNA end at the termination step, and therefore attests to a mechanistic switch from hydrolysis to transesterification. One explanation for the termination without re-initiation is that the hydroxyl group of the new tyrosine may still be able to coordinate a water molecule that attacks the phosphodiester bond at the newly synthesized origin in an analogous manner to the original glutamic acid side chain. These results show that alteration of a key aminoacid side chain in the active site of RepA can convert its mechanism of action from hydrolysis to transesterification. They also imply an evolutionary link between RepA and ΦX174 gene A protein. The two proteins have acquired, through evolution, a different mechanism of action, to fit the requirements of their respective replicons.

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Initiation and Termination of Rolling-Circle Replication of Plasmids in Gram-Positive Bacteria. Saleem A. Khan, Adam C. Zhao and Rais A. Ansari, Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, USA.

pT181 is a *Staphylococcus aureus* plasmid which serves as a prototype for a large number of small, multicopy plasmids found in Gram-positive bacteria that replicate by a rolling circle (RC) mechanism. The replication initiator protein encoded by pT181, RepC, binds to the origin of replication and nicks one strand of the DNA to generate a primer for the initiation of RC replication. The present study was undertaken to identify the DNA sequence requirements for initiation and termination of replication, and the role of RepC in these steps. In vivo and in vitro replication experiments using plasmids containing one wild-type and one mutant pT181 origin showed that an 18-bp sequence containing the RepC nick site was sufficient for termination, whereas a much larger region including the complete RepC binding site was required for initiation. The 18-bp sequence was sufficient for nicking-closing by RepC, showing that this ability of RepC was required but not sufficient for initiation of replication. Several point mutations within the origin were found to abolish initiation but allowed efficient termination. The above results showed that the sequence requirements for initiation are more stringent than those for termination. We have also used an in vitro system to study the conversion of single-stranded plasmid DNA into the double-stranded form. These results have shown that replication of the lagging strand of the DNA from SSO_U-type origins initiates at a single position. On the other hand, initiation from SSO_A-type origins occurs at multiple positions. RepC acts as a dimer in pT181 replication and we have previously identified its DNA binding and topoisomerase domains, both of which are required for its replication activity. Heterodimers of two RepC mutants, one defective in DNA binding and the other in its topoisomerase activity, were generated. In vitro replication experiments demonstrated that while the individual RepC mutants were defective in their replication activity, the heterodimer preparation supported replication to significant levels. These results suggest a role for each of the two RepC monomers in pT181 replication.

Ti plasmid transfer

Chairperson: Anthony P. Pugsley

Wide host range DNA and protein transport mediated by the virulence system of Agrobacterium tumefaciens.

P.J.J. Hooykaas

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The bacterium Agrobacterium tumefaciens introduces a segment of its Ti plasmid DNA (T-DNA) into plant cells, thereby inducing the formation of plant tumors called crown galls (for review: Hooykaas and Beijersbergen, 1994). The T-DNA transfer process requires the function of several proteins encoded by the vir-regulon. One of the early steps in T-DNA transfer is Vir-mediated processing of the T-region resulting in the formation of linear, single stranded molecules called T-strands. Similar molecules are also formed during bacterial conjugation. In order to find more evidence for a relationship between the processes of T-DNA transfer and bacterial conjugation, we studied whether the virulence genes of Agrobacterium could replace the transfer genes of conjugative plasmids and mediate plasmid transfer between bacteria. The virulence genes could indeed mediate inQ plasmid mobilization between bacteria (Beijersbergen et al, 1992). The virB operon genes and the virD4 gene were essential for plasmid mobilization.

Otherwise we studied the host range for T-DNA transfer and found that not only plant cells, but also cells of the yeast Saccharomyces cerevisiae could act as recipients. T-DNA integration was studied both in plant cells and yeast cells in order to determine the importance of host factors (Bundock et al, 1995).

During T-DNA transfer not only a segment of DNA, but also certain Vir-proteins are introduced into the recipient plant cells, These may be involved in transport, protection and integration of the T-DNA.
One of these Vir-proteins is VirF, which is essential for T-DNA transfer to

particular hosts only (for example the plant Nicotiana glauca). We found indications that the VirF protein by itself (i.e. without associated DNA) may be transferred to plant cells during tumor induction by means of the vir-encoded transport system (Regensburg-Tuïnk and Hooykaas, 1993). We concluded that transport systems mediating transfer of DNA and protein may have a common evolutionary origin, especially considering that it is not naked DNA that is transported, but a pilot protein with a covalently attached DNA strand.

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Trans-Kingdom Conjugation between Agrobacterium and the Plant Cell

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Agrobacterium tumefaciens is widely known and exploited by plant biologists in molecular and genetic studies to introduce DNA into plants (reviewed in (1, 2)). Although best known for this practical application, the transfer of DNA from a bacterium to a plant cell involves numerous fundamental biological processes. With regard to the present workshop on DNA transfer mechanisms, the Agrobacterium DNA transfer system is the best example of how widespread generalized mechanisms in prokaryotes can be, since it has evolved to apply a conjugal DNA transfer system to a eukaryotic cell.

There are two genetic regions on the Ti (tumor inducing) plasmid of Agrobacterium required for plant cell transformation. The T-DNA element is the mobile DNA defined and deliminted by two 25 bp direct repeats at its ends. These borders are cis elements that are acted upon by approximately 16 products of the second region, called the virulence (vir) region. In the presence of susceptible plant cells, the expression of the vir region is induced. Soon after vir induction a single stranded (ss) copy of the T-DNA, called the T-strand, is generated (3). The discovery of the T-strand was the first evidence that Agrobacterium might be involved in trans-kingdom conjugation since its DNA transfer intermediate was ssDNA. Strong support of this model derived from experiments showing that the oriT from the conjugative E.coli plasmid, pRSF 1010, can substitute for the T-DNA borders in directing DNA transfer to plant cells from Agrobacterium; this hybrid system required the vir region and the cognate mobilization proteins for the oriT of pRSF1010 (4). Soon after, trans-kingdom conjugation was reported between E.coli and yeast (5).

Regarding Agrobacterium , soon after the discovery of the T-strand, researchers focused on the vir region (reviewed in (1,2)). Two vir products, VirD1 and VirD2, were found responsible for ss nicking and processing at the T DNA border, VirE2 was discovered to be a tenacious, non sequence specific ssDNA binding protein, and the virB operon was shown to encode 11 polypeptides with primary amino acid sequences that predicted localization to the bacterial membranes and periplasmic space. This latter finding prompted speculation that the virB proteins might form an export channel for T-strand exit from the bacterial cell. Parallel research in several labs (reviewed in (6)) provided increasing evidence that T-strand transfer to plant cells and bacterial conjugation were highly homologous processes; for example, the DNA sequences of oriT of plasmid RP4 and the T-DNA borders were similar, and large operons (such as virB and Tra2 (6), IncN (7)) encoded proteins with similar amino acid sequences. Furthermore, these conjugative operons are homologous to proteins of pertussis toxin export system (reviewed in (6)), suggesting there must be some common mechanisms underlying protein and DNA export.

The work in our laboratory for the last 5 years has been focused in two general areas, 1) how does the T-strand enter the plant cell nucleus, and 2) the characterization of the protein products of the virB operon. Regarding the first goal we have evidence that the T strand is not likely to be transferred to the plant cell as a naked ssDNA. Instead, two proteins VirD2 at the 5' end and cooperatively bound VirE2 along its length, form a ssDNA protein complex,

called the T-complex. In support of the T-complex and its role in the plant cell, we have demonstrated that both VirD2 and VirE2 contain functional eukaryotic nuclear localization sequences (reviewed in (1,2)) . Our most recent work demonstrates that in vitro made complexes between fluorescently labeled ssDNA and VirE2 localize to plant cell nuclei following microinjection into the cytoplasm of individual plant cells (8).

Regarding the second goal, we and others have shown that most of the VirB proteins are associated with the bacterial membranes by cell fractionation experiments (9, 10). More recently we have initiated an extensive biochemical study to assay for possible interactions between individual VirB proteins with the aim of revealing the topology of the putative T-complex export channel (11). Antibodies were raised against each of the VirB proteins and were used for analysis of the arrangement of the VirB proteins in the bacterial envelope by (i) membrane fractionation, (ii) crosslinking, (iii) immunoprecipitation, (iv) digestion with proteases, and (v) extraction with detergents. These methods were applied to each of the VirB proteins, and the findings will be summarized. An example of one of the key findings emerging from these studies, is that VirB7 is a lipoprotein, since it labels with 3H-palmitic acid, and it resides preferentially in the outer membrane. Analysis of cell lysates in nonreducing gels in one and two dimensions shows different forms of VirB7, presumably representing monomer, dimer and a higher molecular weight complex.VirB9 was shown to be an interacting partner of VirB7 in the high molecular weight complex; the interaction occurs via a highly unusual inter-molecular disulfide bridge. Thus, VirB7 may serve to anchor the hydrophilic VirB9 in the outer membrane. However, the occurrence of other forms of VirB7 protein suggest that it may have additional function(s) in VirB complex assembly or T-complex export. Biochemical and genetic data from our lab and other groups will be
assembled to a model of VirB complex architecture, and discussed in the context of homologous protein and DNA export systems.

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THE ROLES OF VIRULENCE PROTEINS OF *AGROBACTERIUM* IN TRANSFER AND INTEGRATION OF T-DNA

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Two virulence proteins perform special functions in the target plant. VirD2 is covalently attached to the 5'-terminus of the T-strand while VirE2, a single-stranded, DNA binding protein, coats the T-strand. We had shown previously that the VirD2 protein mediates nuclear targeting of the T-DNA. The involvement of VirD2 in T-DNA integration was investigated with the help of a mutant. This mutation reduced the efficiency of T-DNA transfer, but the efficiency of integration of T-DNA per se was unaffected. Southern and sequence analyses of integration events obtained with the mutated VirD2 protein revealed an aberrant pattern of integration. These results indicate that the wild-type VirD2 protein participates in ligation of the 5'-end of the T-strand to plant DNA and that this ligation step is not rate limiting for T-DNA integration (1, 2).

Different putative roles of the VirE2 protein in the plant cell were analyzed. In the absence of the VirE2 protein mainly truncated versions of the T-DNA were found integrated. This implies that this protein protects the single-stranded T-DNA against nucleolytic attack during the transfer process, indirectly demonstrating that VirE2 protein is interacting with the ss-T-DNA on its way to the plant cell nucleus. Furthermore, it was found that the VirE2 protein is not involved in directing the ss-T-DNA to the plant cell nucleus in an NLS (nuclear localization signal) dependent manner, a function which is carried by the NLS of VirD2. In addition, the efficiency of T-DNA integration into the plant genome was found to be VirE2 independent. Thus the VirE2 protein of *Agrobacterium tumefaciens* is required for the preservation of the integrity of integrated T-DNA but is not essential for the integration step (3).

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THE CONJUGATIVE DNA TRANSFER SYSTEM OF THE Ti PLASMIDS. Stephen K. Farrand. Departments of Crops Sciences and Microbiology, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801, USA.

The Ti plasmids of *Agrobacterium tumefaciens* encode two conjugation systems. One, encoded by the *vir* regulon, is specialized to transfer a specific portion of the Ti plasmid, called the T-region, to plant cells. The second, encoded by the *tra/trb* regulon, mediates transfer of the entire Ti plasmid to a bacterial recipient. *Vir*, at least, is entirely self-contained, and does not depend on functions encoded by *tra/trb*. Interestingly, under the proper conditions, the *vir*-mediated system can transfer plasmids from the donor to bacterial recipients.

Sequence analysis has established that the genes and the *cis*-acting components of the *vir* system share ancestry with conjugal transfer systems of other bacterial plasmids. The T-region border sequences, which are the conceptual homologues of an *oriT*, are related structurally and functionally to the *oriT* of the IncP1α plasmid RP4. Moreover, VirD2, the site-specific endonuclease that nicks at borders, is related to TraI, the *oriT*-specific endonuclease of RP4. The mating bridge assembly, on the other hand, seems to have arisen from a different lineage. The 11 *virB* genes that apparently encode the mating bridge are closely related, at the gene organization and the amino acid sequence levels, to the *tra* genes of the IncN plasmid pKM101. Thus, *vir* apparently is a chimeric conjugation apparatus, containing elements deriving from the same ancestors as those of IncP1α and IncN plasmids.

The Tra system of the Ti plasmid appears also to be chimeric in its ancestry. The genes encoding the Tra functions of the IncRh1 Ti plasmid pTiC58 are organized into three operons. Two of these, comprising the *tra* region, are divergently transcribed from a central 258 bp region that encodes the *oriT* domain. The *oriT* of the Ti plasmid contains a 12 bp region that is almost identical in sequence to the nick site of the IncQ plasmid, RSF1010. However, while the motifs and spacing are similar, the inverted repeats flanking the putative nick site are not related to those of the RSF1010 *oriT*. The two *tra* operons encode a total of six genes, *traAFB*, and *traCDG*, that are involved in conjugal transfer. TraA is a large protein, which by sequence analysis contains two domains. The amino-terminal third of the protein probably encodes the *oriT*-specific endonuclease activity. This domain is related to MobA, the *oriT*-specific single-stranded endonuclease of RSF1010. The middle third of TraA is related to the helicase domain of TraI, the bifunctional *oriT*-processing enzyme from plasmid F. The carboxy-terminal third of TraA is not significantly related to any protein sequences in the databases. While TraC and TraD are not related to any proteins in the databases, TraF and TraG are related to proteins of the same names from the conjugation system of RP4. Genetic analysis indicates that TraG and TraF are required for conjugation. TraB, while not essential for transfer, is required for maximum mating frequencies. Interestingly, *traB* is closely related at the nucleotide sequence level to an open reading frame located in the *vir* region of the octopine-type Ti plasmid pTi15955. The role of this *vir* ORF, if any, has not been determined. Because of potential polar effects of our insertion mutations, we could not determine whether TraA, TraC or TraD are essential. However, based on sequence analysis, we predict that TraA is essential for the processing of the DNA transfer intermediate. Sequence analysis indicates that the *tra* region encodes the DNA metabolism functions of conjugation. However, based on similarities to genes in the RP4 system, two genes in this region, *traF* and *traG* encode products that are themselves probably not involved in DNA processing. TraF from both plasmids has the signatures of a membrane-associated protein. Moreover, with RP4, TraF, while not required for nicking at *oriT*, is required for sensitivity to infection by RP4-specific bacteriophages. TraG, on the other hand, is not required for either process, although it is essential for conjugation.

The third operon of the Ti plasmid, called *trb* is located about 100 kb from *tra*, and maps between *noc*, which encodes transport and catabolism of the opine nopaline, and *oriV/rep*. This 11 kb region encodes 12 genes, 11 of which encode products that show significant sequence

relatedness to the products of the *trb* genes of RP4. Moreover, except for *trbJ*, *traK*, and *trbL*, the gene order of the two sets of *trb* genes is identical. Insertion mutations throughout *trb* abolish conjugal transfer of the Ti plasmid. The *trb* regions of the two plasmids are believed to encode the mating bridge assemblies. Furthermore, *trb* of RP4, along with *traF* confers sensitivity to male-specific bacteriophages. Despite its similarity to *trb* of RP4, the Ti plasmid Tra system does not confer sensitivity to RP4-specific phages.

Sequence analysis indicates that components of the Ti plasmid conjugation system share phylogenetic ancestry with those of several conjugal plasmids. The DNA metabolism functions including the *oriT* and the amino-terminus of TraA, the corresponding endonuclease, clearly are related to those of the IncQ plasmids. However, unlike MobA of RSF1010, TraA contains a putative helicase domain which is related to that of TraI from the IncF transfer system. The mating bridge assembly quite clearly derives from a third ancestor, that which also gave rise to the mating bridge of the IncP1α plasmids. Furthermore, like RP4, one gene required for the mating bridge, *traF*, is located in the *tra* regulon, rather than in *trb*.

The *tra* and the *trb* regions of pTiC58 are necessary and sufficient for conjugal transfer. We have reduced the system to a set of two binary plasmids, one encoding *tra*/*oriT*, the other the *trb* region. An *Agrobacterium* donor harboring both plasmids transfers the *tra*/*oriT* component to recipients at a high frequency. Donors harboring one or the other of the two components show no transfer. Thus, the Tra system is completely independent of the *vir* system, a conclusion consistent with the sequence analysis that suggests that the two systems are phylogenetically distinct, and most probably arose independently.

Despite the similarities in their *oriT* regions, the Ti plasmid does not mobilize transfer of RSF1010. On the other hand, RP4 mobilizes RSF1010 despite the fact that the *oriT* regions of the two plasmids are not related. In conjugal systems, TraG or its cognate homologue is believed to interface the relaxosome with the mating bridge. RSF1010 does not encode its own TraG, or a mating bridge, and evidently parasitizes these functions from the mobilizing plasmid. Indeed, the Ti plasmid Tra system mobilizes pBS141, a derivative of RSF1010 in which *traG* of RP4 has been cloned. Mobilization is dependent upon *traG*: mutations in this gene that abolish transfer by RP4, similarly affect the ability of the Ti plasmid to transfer the recombinant plasmid. These results suggest that while TraG of the Ti plasmid recognizes its own relaxosome and mating bridge, it does not recognize the relaxosome of RSF1010. On the other hand, TraG of RP4, which recognizes the relaxosomes of RP4 and of RSF1010, also evidently recognizes the Ti plasmid mating bridge.

Finally, in addition to transferring T-strands, the *vir* system also mobilizes RSF1010 both to plant and bacterial recipients. Interestingly, transfer of the T-strand by *vir* is inhibited when RSF1010 is present in the donor. Conjugal transfer of the Ti plasmid, on the other hand, is not inhibited by RSF1010 or by pBS141, even under conditions in which the Ti plasmid Tra system mobilizes transfer of the IncQ plasmid. This suggests that there may be fundamental differences between the Tra- and *vir*-mediated DNA transfer processes.

Conjugative transfer

Chairperson: Patricia Zambryski

Initiation and Termination of Conjugative DNA Transfer

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The relaxase (TraI) of the conjugative broad host range plasmid RP4 (IncPα) plays a key role in initiation and termination of transfer DNA replication that takes place during transmission of the plasmid from a donor to a recipient cell. TraI functions as a DNA strand transferase that cleaves a unique phosphodiester bond at the *nic* site of the transfer origin (*oriT*) [1]. The cleavage reaction consists in a reversible transesterification reaction leading to transfer of the 5' phosphoryl at *nic* to the hydroxyl group of TraI Tyr22 [2]. Hence, cleavage results in the covalent attachment of TraI to the 5'-terminus of the plasmid strand destined for transfer.

To investigate the protein's ability to function in a "second cleavage" reaction (Fig. 1) [3] proposed to terminate rolling circle mode transfer DNA replication, single- stranded oligonucleotides containing the *nic* region were immobilized at their 3' ends on ferromagnetic beads and cleaved by TraI. The resulting covalent TraI-oligonucleotide adducts were isolated by magnet separation, and in a second step incubated with 5' labeled oligonucleotides containing the *nic* region. The resulting covalent TraI-oligonucleotide adducts were active in the joining reaction but unable to cleave oligonucleotides containing an intact *nic* region, indicating that "second cleavage" probably requires a TraI dimer, since a monomer is insufficient. The covalently attached oligonucleotide determines the affinity of the relaxase for the 3' terminus of the T-strand, indicating that the protein-bound 5' terminus of the T-strand plays an important role in the recognition/binding and probably in the adjustment of the 3' end of the T-strand in the active center of the enzyme.

Alignment of the TraI amino acid sequence with relaxases from several origins resulted in the identification of three conserved motifs. Invariant amino acid residues within these motifs were the targets for site-directed mutagenesis of TraI [4]. The resulting mutant proteins were tested *in vivo* and *in vitro* for their activities, cleavage of single- and double-stranded substrates, and enhanced topoisomerase activity [4,5]. The resulting phenotypes allowed us to allocate particular relaxase functions in distinct TraI domains, e.g. substrate recognition or activation of the reactive Tyr22. To further the biochemical characterization of TraI mutants, the DNA binding ability of TraI derivatives immobilized on magnetic beads were investigated. Following incubation with substrate oligonucleotides, magnet separation in combination with gelelectrophoretic analysis of reaction products in the supernatant, allowed us to quantify oligonucleotides non-covalently bound to TraI. We showed that amino acid residues in each relaxase motif are involved in addition to their function in catalysis in substrate binding.

To uncouple substrate binding and cleavage-joining we applied partially biotinylated TraI mutant proteins that were immobilized to magnetic beads. Using this approach we could demonstrate that tight DNA substrate binding and cleaving-joining are independent processes. Enhanced topoisomerase activity of some TraI mutants [5] was correlated with low specific substrate binding affinity in conjunction with high cleaving-joining activity.

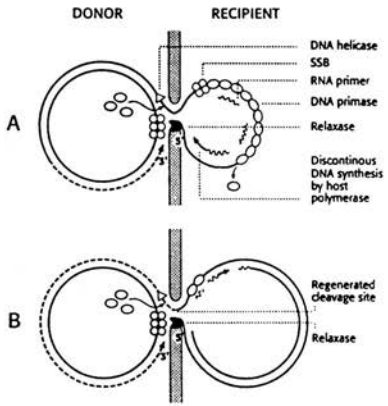

Fig. 1. Schematic representation of two stages in the processing of DNA during conjugative transfer of plasmids. Panel A shows transfer of the preexisting DNA strand (heavy continuous line) with a 5' to 3' polarity. The relaxase (TraI) is covalently linked to the unique 5' terminus at the cleaved *oriT* site, and the complex is located close to the DNA transport pore. The transferring strand (T-strand) is escorted into the recipient cell by plasmid DNA primase (TraC). RNA primers are elongated by the replicative DNA polymerase of the host (*E.coli* DNA polymerase III holoenzyme). In the donor cell, DNA unwinding is mediated by a plasmid or host-encoded DNA helicase yet to be identified; here the helicase is translocating processively in the 5' to 3' direction on the bound DNA strand. Synthesis of the replacement strand (broken line) by a rolling-circle mode of DNA replication has reconstituted an *oriT nic* region in the donor. Small circles indicate single-stranded DNA binding (SSB) protein. No attempt has been made to indicate the complexity of the cell envelopes (hatched barrier between cells) or the DNA transport pore. Panel B shows the stage immediately prior to circularization of the transferred DNA strand by the "second cleavage" mechanism. DNA transfer has brought the reconstituted *nic* region in single-stranded form into close proximity with the relaxase-linked to the 5' terminus of the T-strand. Relaxase cuts the reconstituted *nic* region and joins the 5' and 3' termini to give a monomeric circle of transferred DNA. Circularization is occurring before the completion of complementary strand synthesis in the recipient cell.

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CONJUGAL DNA METABOLISM OF INCW PLASMID R388

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IncW plasmids promiscuously transfer DNA with high efficiency to a variety of gram-negative bacteria, yet their DNA transfer regions are among the shortest known to date. Thus the transfer machineries of these plasmids are both simple and efficient, which makes their study most interesting to elucidate the basic mechanisms of bacterial conjugation.

We are studying the IncW plasmid R388, whose transfer genes are included in a contiguous DNA region of 15 Kb (1). For conjugal mobilization, the plasmid requires the oriT and three adjacent genes, named trwA, trwB and trwC (2). The rest of the transfer region is involved in cell-to-cell contact functions.

TrwA is not absolutely essential for conjugation, but in its absence transfer frequencies drop 1000-fold (2). It shares about 20% identity with conjugal proteins NikA of IncI plasmid R64 and TraJ of IncP plasmids RP4 and R751. These proteins are involved in the initial nicking reaction at oriT, assisting the oriT endonuclease. However, we have not detected any involvement of TrwA in the R388 oriT nicking reaction. We have purified TrwA from an overproducing strain and by crosslinking studies we have determined that it forms tetramers in solution. Gel shift assays and footprinting studies have shown that TrwA binds to four repeated sequences at one end of R388 oriT, in a region overlapping the trwA promoter. Conjugation assays using oriT deletions show that TrwA binding to this region is required in vivo for DNA transfer (3). The TrwA binding pattern reminds of that of TraK of IncP plasmids or TraM of IncF plasmids (4,5), so TrwA could also play a role in conjugation by changing the oriT DNA topology upon binding.

TrwB belongs to the VirD4 family of proteins (6), which are essential conjugal factors with a putative ATPase domain and several trans-membrane domains. In analyzing TrwB, we have extended our studies to other members of this family to determine the presumably common role they play in conjugation. It has been determined that these proteins are required, together with the pilus functions, to mobilize RSF1010, ColE1, or ColE3 (7, 8, 9). The mobilizable plasmid CloDF13, on the contrary, does not require a VirD4-family member to be mobilized (9); this plasmid codes for a protein, MobB, which is also a VirD4-homolog. We believe that this family of proteins plays a role in the connection of the relaxosome with the membrane pore. In fact, mobilization assays shuffling different types of pilus genes and VirD4-homologs suggest

that mobilization frequencies are affected by the interactions of the VirD4-homolog with both the relaxosome and the pilus proteins (9). Given that TrwB can be complemented by RP4 TraG for ColE1 or RSF1010 mobilization but not for R388 conjugation, we constructed mutations along trwB in search for TrwB mutants that were impaired in one of the two functions, with no success (9); this implies that TrwB works similarly for conjugation and mobilization. Probably mobilizable plasmids have evolved to be more flexible for their coupling factor.

TrwC plays two roles in R388 conjugation: it is a DNA helicase (10) and the oriT-specific endonuclease (11), showing in vitro nicking/closing activity on oligonucleotides spanning the nic site (12). TrwC is homologous to TraI of F, an homology that is also present between their respective oriT target sequences (2). Both proteins differ from the rest of oriT endonucleases in their DNA helicase activity and in their ability to nick double-stranded oriT DNA in vitro in the absence of any other protein (11,13). The first 272 amino acids of TrwC are sufficient to catalyze oriT-specific recombination events which presumably reflect the nicking-closing activity of the enzyme (14). We have dissected TrwC by overexpressing and purifying different segments of the protein and assaying their DNA helicase, ATPase, oriT nicking and oligonucleotide nicking-closing activities (12). As expected, the ATPase and DNA helicase activities lie in the carboxy-terminal part of TrwC together with the dimerization domain, and the nicking-closing activity is conserved in the amino-terminal 275 amino acids. However, this peptide was not able to nick oriT-containing plasmids while another containing the first 348 amino acids of TrwC was. Thus the ability to cut and seal single-stranded DNA is not enough to act on double-stranded substrates but an additional segment of the protein provides this proficiency. We propose that this region might mediate TrwC binding to supercoiled DNA. So we have delimited the small region that distinguishes functionally TrwC and F-TraI from the rest of conjugal endonucleases known to date.

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Reconstitution and Biochemical Characterization of the F Plasmid Relaxosome *in vitro*; Steven W. Matson, Michael T. Howard, William C. Nelson and Jon A. Sherman; Department of Biology, University of North Carolina, Chapel Hill, NC 27599 USA

A central step in the transfer of genetic information during bacterial conjugation involves the formation of a protein-DNA complex, called the relaxosome, at the origin of transfer (*oriT*). During conjugation, the relaxosome introduces a site- and strand-specific nick from which the physical transfer of a single strand of DNA is initiated. Genetic studies utilizing the *E. coli* F plasmid have shown nick formation to be dependent on the products of two F-encoded genes, *traI* and *traY* (1). Purified TraIp (also known as DNA helicase I) has long been known to contain DNA helicase activity (2). Presumably unwinding of the F plasmid, catalyzed by TraIp, provides the single-stranded DNA that is transferred to the recipient cell. In addition, previous studies from this laboratory have shown that highly purified TraIp also nicks *oriT* in a site- and strand-specific manner (3). Biochemical characterization of the reaction that produces the nick at *oriT* has revealed that this reaction is, in fact, a transesterification reaction in which TraIp is covalently bound to the 5'-end of the nicked DNA strand (4). Phosphodiester bond energy is preserved in the covalent linkage of DNA to protein, and TraIp is also able to catalyze the religation of the nicked strands (5). However, at low ionic strength the nicking reaction is independent of the addition of TraYp, and thus does not accurately reflect the *in vivo* protein requirements.

The *traY* gene product, TraYp, is the second F-encoded gene product implicated in nick formation. TraYp is a site-specific DNA binding protein with two known specific binding sites on the F plasmid (6). One binding site, *sbyA*, is located within *oriT* approximately 60 base pairs from the site that is nicked to initiate conjugative DNA strand transfer. The second binding site, *sbyB*, is coincident with the mRNA start site for the TraYI operon. The role of TraYp bound at this site is unknown. In addition, analysis of the *oriT* region has revealed two binding sites for the host protein integration host factor (IHF). One IHF binding site, IHF A, is located between the nick site and the TraYp binding site. The second IHF binding site, IHF B, is located distal to the TraYp binding site (7).

To explore possible protein-protein interactions occurring at *oriT*, highly purified TraIp, TraYp and IHF were incubated with a supercoiled *oriT*-containing DNA substrate. Under conditions where the basal TraIp-catalyzed nicking reaction was nearly undetectable (i.e. at higher ionic strength) a marked enhancement of the nicking reaction catalyzed by TraIp was observed in reactions containing both TraYp and IHF (8). High concentrations of either protein failed to substitute for the absence of the other protein. In addition, TraIp was able to nick a linear *oriT*-containing DNA substrate when both IHF and TraYp were present in the reaction; this DNA substrate is not nicked by TraIp alone under any conditions we have tested. Individual protein concentration requirements for the

supercoiled and linear nicking reactions were similar, and the reactions occurred at equal velocities suggesting the they are biochemically identical. Concentrations of TraYp and IHF that yield half-maximal activity in the nicking assays compare well with the reported K_d values for IHF and TraYp binding sites in *oriT*. These data suggest that TraYp and IHF bind to their respective binding sites in *oriT* independent of one another. Studies performed using *oriT* deletion mutants indicate that the second IHF binding site (IHF B) and the binding site for TraMp are not required for the nicking reaction. Thus reconstitution of an active relaxosome at oriT involves the binding of TraYp to *sbyA*, the binding of IHF to IHF A, and the binding of TraIp at the nick site. The addition of TraMp has no effect on this reaction.

To further characterize this reaction we have used DNase I protection experiments and electron microscopy to investigate the mechanism of relaxosome formation (9). Order of addition experiments have revealed that IHF and TraYp must be present in the reaction mixture, with an *oriT*-containing DNA substrate, prior to the addition of TraIp. These results suggest that IHF and TraYp form a complex at *oriT* prior to TraIp binding. DNase I footprinting studies indicate that IHF and TraYp bind their respective binding sites independently. TraIp fails to bind *oriT*-containing DNA in the absence of IHF and TraYp. However, the addition of IHF, TraYp and TraIp, in that order, produces a region of DNase I protection that extends across the nick site within *oriT*. Thus, TraYp and IHF form a protein-DNA complex that facilitates the binding of TraIp to assemble a relaxosome capable of introducing a site- and strand-specific nick at oriT. Interestingly, the DNA does not seem to be wrapped around the proteins in this complex as there is no linear foreshortening of the DNA within the complex as measured from electron micrographs.

The binding of TraYp to *sbyA* is a critical step in the formation of the relaxosome at *oriT*. To further understand this interaction we have explored the binding dynamic of TraYp to the two known specific binding sites on the F plasmid (10). The sequence of TraYp exhibits a unique direct repeat structure that is predicted to have a ribbon-helix-helix DNA binding motif in each repeat unit. The stoichiometry of TraYp binding to DNA has been determined to further support the hypothesis that TraYp is a member of the ribbon-helix-helix family of DNA binding proteins. A glutathione-S-transferase-TraY fusion protein (GST-TraYp)was purified and shown to possess nearly wild-type DNA binding activity. DNA binding experiments were performed in which a DNA ligand containing *sbyA* was incubated with either the fusion protein, the wild-type protein or both proteins. We observed five distinct bound species in a polyacrylamide gel mobility shift DNA binding assay: TraYp monomer bound, TraYp dimer bound, GST-TraYp monomer bound, GST-TraYp dimer bound and a bound heterodimer. These results indicate that TraYp can bind DNA as a monomer or a dimer. Thus a TraYp monomer folds into a stable three-dimensional structure similar to that of the ribbon-helix-helix proteins Arc or Mnt. A homology model of a TraYp monomer has been constructed using the co-crystal structure of Arc bound to DNA as a template to provide additional support for this conclusion. The sequence of amino acids between the two direct

repeats in TraYp are able to bridge the distance from the end of one repeat unit to the beginning of the second repeat unit without significant perturbation of the structure at either end of this region. Moreover, TraYp will dimerize upon binding to DNA in a manner similar to the way other ribbon-helix-helix proteins tetramerize on DNA. We have also shown, using *oriT* deletion mutants, that a monomer of TraYp will bind a half-site deletion of the *sbyA* binding site. Of particular interest is the fact that a monomer of TraYp bound to this half-site deletion mutant is still able to stimulate the nicking reaction catalyzed by TraIp. Thus a TraYp momomer bound to *sbyA* in *oriT* is sufficient to fulfill the role of TraYp in initiating conjugative DNA strand transfer.

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Requirements for Nicking at F and F-like Transfer Origins *in vivo*

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In order to better understand the conditions that induce conjugative plasmids to transfer as well as those which stimulate bacterial cells to engage in conjugation we have developed an *in vitro* technique for monitoring transfer expression-dependent *in vivo* nicking at the *oriT* of conjugative plasmids. The assay has been developed using F-like plasmids as a model. The specificity of the *in vitro* reaction product as an indicator of the abundance of conjugative plasmids cleaved at *oriT in vivo* has been confirmed according to several criteria.

Using this technique we found that detection of *in vivo* nicking activity did not require bacterial conjugation. Plasmids F, R1-19 and R1-16, all derepressed for *tra* operon expression, but not R1 which is *fin*+, were nicked *in vivo* with an apparently high efficiency even when the host cell was not exposed to a recipient strain. Thus, the initiation of transfer of derepressed plasmids does not require as an initial step the synthesis and assembly of the components required for cleavage at *oriT*.

pOX38 derivatives of F carrying insertion mutations in genes *traQ* (pilus minus, conjugation minus)[1] and *traX* (pilus defective, conjugation reduced)[2] were observed to be nicked *in vivo* with the equivalent efficiency as pOX38. The presence of a functional pilus is, therefore, not a requirement for nicking at *oriT*. This finding suggests the possibility that no feedback mechanism exists to negatively regulate relaxosome activity although a defect in a plasmid transfer function will ultimately prevent the host cell from conjugating.

The assay was also used to reassess the requirement for specific *tra* gene products for nicking activity *in vivo*. For these experiments we used an *E. coli sfrA* (sex factor regulator)[3,4] deletion strain[5] where *tra* operon expression is so diminished that no nicking of R1-16 is detectable. The R1 *traI* gene was cloned and moved to an inducible expression vector. Uninduced expression of the *traI* gene *in trans* restored nicking at the R1-16 *oriT* in *sfrA* cells. TraI alone is therefore probably sufficient to catalyze nicking *in vivo*. The further involvement of TraY and TraM will be discussed.

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Mechanism of transfer of the conjugative transposon Tn*916*

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The 18-kb transposon Tn*916*, first detected in the chromosome of *Enterococcus faecalis* strain DS16, is the prototype of a closely related family of conjugative transposons widespread in Gram-positive cocci[1,2]. Transposable elements belonging to this family are large DNA segments (≥18 kb) and all contain the tetracycline resistance determinant *tet(M)* alone (e.g., Tn*916*), or in association with other resistance genes (e.g., Tn*3703* and Tn*1545*). Transposition of these elements proceeds by excision to a free, non-replicative, covalently closed circular intermediate that is a substrate for integration. The integration-excision system of these elements is structurally and functionally related to that of lambdoid phages[3,4]. Excision and integration occur by reciprocal site-specific recombination between non homologous sequences of 6 bp. Excisive recombination requires two transposon-encoded proteins, an excisionase (Xis-Tn) and an integrase (Int-Tn), whereas integration requires only Int-Tn. However, as in the λ site-specific recombination system, Int-Tn alone can catalyze excision but this activity is greatly stimulated by Xis-Tn. It is thus likely that the direction of recombination (integration *versus* excision) is determined by the relative amounts of the two proteins in a cell.

Conjugal transfer of Tn*916* and related elements can be viewed as a transposition event involving donor and recipient replicons that happen to be present in different cells and was therefore referred to as conjugative transposition. This event involves circularization of the element in the donor, and transfer of the non-replicative intermediate by conjugation to the recipient where it integrates[5]. Bacterial hosts such as *Lactococcus lactis* and *Streptococcus pneumoniae* in which Tn*916* can not excise are unable to promote conjugative transposition. Thus, in addition to their transfer functions, transfer of this class of elements requires transposon-encoded excision and integration functions. The complete nucleotide sequence of Tn*916* has been determined[6], which enabled the location of a functional *oriT*[7] and the identification of some ORFs displaying significant homology with other known proteins involved in conjugation (antirestriction protein Ard of ColIb-P9, MbeA mobilization protein of ColE1).

The frequency of conjugative transposition of Tn*916* between isogenic strains of *E. faecalis* may range over more than 4 orders of magnitude between different Tn*916*-containing donors and is not related to the number of transposon insertions in the donor

strain, although strains with a high donor potential generally contain multiple insertions. The host range of transfer of Tn*916* might depend on the donor potential of the transposon-delivery strain: only strains having a high donor potential can undergo conjugative transposition from Gram-positive to Gram-negative bacteria[8]. Tn*916* insertions located at different sites of the bacterial genome might possess different donor potentials when considered independently and it has been suggested that the insertion with the greatest potential would be the most likely to excise initially and that this event would stimulate, by *trans*-activation, movements of all other transposons present in the cell[9]. Dosage of Tn*916* circular intermediates in *E. faecalis* by using a quantitative PCR assay has revealed that Tn*916* conjugation frequency was dependent on the copy number of circular intermediate present in the donor cell[10]. Thus, excision appears to be the initial and rate-limiting step of both the intra- and intercellular mobility of this class of elements. We recently constructed an excision-reporter plasmid to characterize, qualitatively and quantitatively, the intracellular mobility of Tn*916* in Gram-positive bacteria with different donor potential. Our results are not consistent with the mechanism of *trans*-activation described above nor with the proposal that there is a linear relationship between excision and transfer. They suggest that differences in donor potential of transposon-delivery strains are due to the fact that the transfer functions of inserted transposons might be expressed at different levels depending on their insertion sites.

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Structure and Function of Helicases

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Several biologically important processes like DNA replication, repair, recombination and bacterial conjugation require melting of double helical DNA into single strands (ssDNA)[1,2] This is accomplished by a ubiquitous family of enzymes called helicases which disrupt the Watson-Crick hydrogen bonds and are fueled by nucleoside-5'-triphosphates (mainly ATP). Helicases are highly processive; some operate in 5'-to-3', some in 3'-to -5' direction, and one is known to operate in both directions. Besides DNA, helicases are also found which unwind double stranded RNA and RNA-DNA hybrids. In *E.coli*, 10 helicases have been isolated so far which are probably required for different DNA unwinding processes.

Helicases bind to ssDNA, on the replication fork, or (more rarely) on blunt ends of DNA. After DNA unwinding, ssDNA binding proteins cover the newly formed single strands. In replication, helicases are associated with DNA polymerase (on the leading strand) and with primase (on the lagging strand).

The molecular weights of helicases are between 30 and 200 kDa. Some of the known helicases are mono - or dimeric, and most of them are hexameric. Thus far, no crystal structure is available, but several of the hexameric helicases have been studied by electron microscopy: *E.coli* helicase DnaB[3]; bacteriophage T7 helicase/primase[4]; *E.coli* RuvB which is part of the SOS repair system[5]; RepA from the plasmid RSF 1010[6] and the *E.coli* RNA-DNA helicase Rho[7]. DnaB and Rho occur as hexamers with threefold rotation axis and D_3 symmetry (trimer of dimers). RuvB as a dimer of hexamers with sixfold rotation axis and D_6 symmetry, the T7 helicase/primase is a two-tiered hexagon, and RepA is a simple hexagon; for the latter, we have obtained single crystals and the X-ray structure determination is under way.

As shown for RuvB and T7 helicase/primase, the ssDNA passes through the central cavity of the hexagon, and other schemes of ssDNA/RNA binding have been proposed for Rho. For unwinding, a „passive" mechanism has been proposed where helicase translocates along

ssDNA and unwinds the duplex. In more recent papers[8,9] an „active" mechanism has been formulated for E.coli Rep helicase during which Rep binds simultaneously to ssDNA and duplex DNA and acts in a „rolling" scheme.

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MECHANISM OF RUVAB-PROMOTED DNA STRAND EXCHANGE.

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The RuvA and RuvB proteins of E. coli, which are induced in response to DNA damage, play important roles in the formation of heteroduplex DNA during genetic recombination and related recombinational repair processes. Together, RuvA and RuvB promote branch migration, an ATP-dependent reaction that increases the length of the heteroduplex DNA. Our electron microscopic studies showed that RuvB interacts with duplex DNA by forming hexameric rings encircling DNA molecules. We have visualized RuvAB complexes with Holliday junctions by electron microscopy and observed the formation of a tripartite protein complex in which RuvA binds the crossover and is sandwiched between two hexameric rings of RuvB. We propose a molecular model for branch migration, a novel feature of which is the role played by the two oppositely-oriented RuvB ring motors. The two ring motors, which are diametrically opposed across the Holliday junction, each encompass a DNA duplex and drive branch migration by catalyzing the directional unwinding and helical rotation of DNA. These studies provide new insight into the mechanism of DNA branch migration during genetic recombination and DNA repair.

Phage DNA extrusion and other processes related to conjugation

Chairperson: Richard P. Novick

Filamentous phage assembly: variation on a pilus theme?

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The filamentous phage constitute a large family of bacterial viruses with single-stranded, circular DNA genomes that infect a variety of Gram negative bacteria. The DNA is extended along the axis of the particle with the packaging signal, a double-stranded "hairpin", located at one end of the filamentous tube which is formed by many copies of the major coat protein. A few copies each of several minor coat proteins are located at the particle ends. The packaging signal end contains two proteins necessary for efficient particle assembly, while the other end contains two proteins required for particle stability and phage infectivity. The virus particles are flexible rods about 65 Å in diameter, similar to the diameters of bacterial pili. The very similar phages M13, fd and f1 use F-pili as a receptor to infect *E. coli*, and other filamentous phages use other conjugative pili. Filamentous phage do not kill their host; rather they are continually extruded or secreted across the bacterial inner and outer membrane without causing cell lysis as the infected cells continue to grow and divide. It is this property - and their filamentous morphology - that distinguishes them from most other bacterial viruses which accomplish their release from the host cell by lysing it.

Two phage-encoded proteins which are not part of the virus particle, the inner membrane pI and the outer membrane pIV, are required for phage assembly. Similarities between these proteins and bacterial proteins from a large number of bacterial pathogens suggest that filamentous phage assembly/export may occur by a mechanism related to that of Type IV pilus assembly and extra-cellular protein export.

Based primarily on genetic evidence, pI plays multiple roles in assembly, potentially interacting with the packaging signal (to initiate assembly), with *E. coli* thioredoxin and one or more coat proteins (to elongate the particle), and (via their periplasmic domains) with the morphogenetic protein pIV to accomplish their assembly and export from the cell. *In vivo* and *in vitro* data support the proposal that pIV forms a large channel through which the assembling phage passes. Such a channel

would have to be larger than previously characterized outer membrane channels in order to accomodate the 65 Å diameter of an assembling phage. Several observations suggest that the putative pIV channel is gated. A model that couples opening of the pIV channel to initiation of phage assembly accounts for many of the known properties of.this specialized export system.

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Over and out: protein secretion in E. coli.

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The general secretory pathway (GSP) in Gram-negative bacteria comprises a universal, Sec-protein-dependent initial step by which locally unfolded proteins are translocated across the cytoplasmic membrane, followed by one of several different terminal steps which direct proteins into the outer membrane or across this membrane into the external medium. The main terminal branch (MTB) of the GSP is used by a wide variety of Gram-negative bacteria to secrete numerous proteins, including toxins and hydrolytic enzymes (2). Proteins secreted by the GSP-MTB fold in the periplasm before they are transported across the outer membrane (1). Our recent studies of a representative of the GSP-MTB, the pullulanase secretion pathway of Klebsiella oxytoca have led to the identification of two regions in the secreted enzyme which, together, are able to promote the secretion of a heterologous protein (unpublished data). The possibility that these two segments of pullulanase could constitute a secretion signal is under investigation.

At least 14 proteins are specifically required in the MTB of the GSP (2). One of these proteins (S) was recently shown by us to be required for the insertion of one of the other MTB components (protein D) into the outer membrane (3), apparently by binding to a periplasmic assembly intermediate (unpublished results). Protein D is of particular interest since it is the only one of the 14 MTB components that is an integral outer membrane protein, and thus the only one likely to form a channel in the membrane to permit the extrusion of the secreted protein. Besides interacting with protein S, protein D forms large multimeric complexes which resist separation and denaturation in SDS (3), which has allowed us to purify small amounts of the protein to homogeneity. Other MTB components share sequence similarity to the subunits of type IV pilins, while still other MTB components are homologous to proteins required for type IV pilus assembly, including the prepilin peptidase with processes and N-methylates type IV pilin precursors. The pilin-like components of the MTB form multimers, but it is not yet clear whether these structures resemble rudimentary pili. The possibility that these pilin-like proteins form part of the outer membrane secretory channel is being investigated (4 and unpublished data).

It is accepted dogma that the laboratory E. coli K-12 strain does not secrete extracellular proteins. It was therefore surprising that homologues of the 12 of the 14 genes coding for the MTB components of K. oxytoca are present in the E. coli K-12 chromosome, that their organisation has been preserved and that at least two of them encode potentially functional proteins (unpublished data). However, the E. coli genes are expressed at extremely low levels under laboratory conditions (unpublished data), raising the possibility that their expression might respond to environmental signals and that E. coli K-12 can indeed secrete protein(s) under certain conditions.

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Structural features of adhesive organelle development in pathogenic bacteria

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We have blended a powerful genetic system with X-ray crystallography, protein chemistry and carbohydrate chemistry to study an advanced macromolecular assembly system: the development of adhesive surface fibers called pili in pathogenic bacteria. We have studied the function of pili in mediating interactions at the host-pathogen interface and the strategies used by pathogenic bacteria to present microbial adhesins in pilus fibers where they are available to mediate attachment to specific receptors on host cell surfaces. Genes important in pili biogenesis, PapA-K, are linked in an operon and their expression coordinately controlled. Pilus subunits are secreted across the cytoplasmic membrane and form assembly-competent complexes with immunoglobulin-like periplasmic chaperones. We investigated how the immunoglobulin-like structures of these chaperones provide a template for the correct folding and import of subunits into the periplasmic space by combining biophysical and genetic approaches with biochemistry and high resolution electron microscopy. This approach has already revealed a conserved anchoring mechanism in the chaperone cleft and a molecular zipper that PapD utilizes to bind subunits. The crystal structure of PapD bound to a PapG peptide provided a "snapshot" of a fundamental process in bacterial pathogenesis: the interaction of an adhesin with a chaperone, which is a prerequisite to adhesin presentation on the microbial surface. Chaperone-subunit complexes are targeted to specific outer membrane assembly sites comprised of proteins called molecular ushers. We studied how the usher interacts with chaperone-subunit complexes to mediate chaperone release and to allow incorporation of subunits into the pilus fiber across the outer membrane, in a specific order determined via complementary surfaces on each subunit type. This process results in composite fibers consisting of homopolymeric rods joined end to end to flexible adhesive fibrillae. The distal location of the adhesin, PapG, at the tip of the adhesive fibrillae, maximizes its ability to recognize Galα(1-4)Gal-containing glycolipid isoreceptors in the host, determining tissue tropism. Chaperone inhibitors are being developed as part of a therapeutic strategy to inhibit attachment of microbial pathogens by blocking pilus assembly and adhesins are being used as new targets in the design of vaccines against bacterial infections.

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Transport Proteins Involved in the Secretion of Pertussis Toxin from *Bordetella pertussis*: Relationship with DNA Transport Proteins. **Drusilla L. Burns, Center for Biologics Evaluation and Research, FDA, Bethesda, MD.**

Pertussis toxin (PT) is an important virulence factor produced by *Bordetella pertussis*, the causative agent of the disease pertussis (whooping cough). The toxin has an A-B structure, *i.e.* it is composed of an enzymatically active subunit, the S1 subunit, and a binding component known as the B oligomer. The B oligomer is made up of subunits S2, S3, S4, and S5 found in a 1:1:2:1 ratio. These subunits form a ring-like structure upon which the S1 subunit sits. The B oligomer contains the binding site for the eucaryotic cell receptor as well as a binding site for ATP which regulates the activity of the toxin. Once the toxin reaches the target eucaryotic cell, the S1 subunit catalyzes the ADP-ribosylation of a family of GTP-binding regulatory proteins which are involved in signal transduction. ADP-ribosylation of these proteins by PT inhibits signal transduction resulting in a number of biological consequences.

In order for the toxin to exert its action on eucaryotic cells, it must be secreted from the bacterial cell after it has been synthesized and assembled. The question arises how this large toxin (M_r = 105,000) might cross the inner and outer membrane barriers of *B. pertussis*. Each of the PT subunits is synthesized with a signal sequence raising the possibility that the subunits may cross at least the inner membrane individually, in the unassembled state. However since only the holotoxin form of the molecule is secreted (1), the assembled toxin must cross at least one membrane barrier.

Recently, we found a set of genes, termed *ptl* genes, which are necessary for secretion of PT from *B. pertussis* (2). The *ptl* genes are located directly downstream from the *ptx* genes which encode the structural subunits of the toxin. Evidence has been obtained which suggests that the *ptx* and *ptl* genes are expressed from a single promoter (3).

The *ptl* region contains nine genes, *ptlA-ptlI*. Each of the Ptl proteins is predicted to share homology with one of the VirB proteins of *Agrobacterium tumefaciens*, which are necessary for the transport of T-DNA across the bacterial membranes. The Ptl proteins also share homology with proteins of several conjugal transport systems including the Tra proteins of the IncN plasmid pKM101 and the conjugal transport proteins of incompatibility groups W and P (4-6). The *ptl* genes are colinear with both the *virB* genes of *A. tumefaciens* and the *tra* genes of pKM101. The Ptl proteins include homologs of every essential VirB protein except for VirB5 and every essential Tra protein of pKM101 except for TraC.

The functions of the individual Ptl proteins remain unknown. Three of the Ptl proteins (PtlE, PtlF, and PtlG) have been visualized using antibodies raised to proteins comprised of maltose binding protein fused to the individual Ptl proteins (7). All three of these Ptl proteins were localized to the membranes of *B. pertussis* as might be expected if the proteins were to form a transport pore or channel through which the toxin is exported. Two Ptl proteins, PtlC and PtlH, have sequence motifs characteristic of nucleotide binding sites (8). Therefore, these

proteins might provide energy for secretion or may act as proteins which signal the opening or closing of a transport gate or channel.

Thus, our results suggest that PT utilizes a protein export system to exit the bacterial cell which is similar to a number of systems used by a bacteria for transport of DNA across their membranes.

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Transposition

Chairperson: James C. Wang

The Molecular Machinery of Mu Transposition

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The tetrameric Mu transposase promotes two DNA cleavage and two DNA joining (strand transfer) reactions to catalyze transposition. We are investigating the regions of transposase involved in cleavage and strand transfer and the architecture of the tetrameric protein complex. The core domain of transposase participates directly in catalysis of both cleavage and strand transfer. Part of this domain is structurally related to the catalytic cores of HIV and ASV integrases (Rice and Mizuuchi, 1995; Dyda et al., 1994; Bujacz et al., 1995) as well as regions of RNase H and the Holliday junction resolving enzyme RuvC (reviewed in Grindley and Leschziner, 1995). Sequence alignments indicate that similar catalytic regions are wide spread among recombinases. Three acidic amino acids in the structurally related region are especially important for catalysis; even conservative substitutions at these positions severely compromise both the cleavage and strand transfer activity (Baker and Luo, 1994; Kim et al., 1995). Biochemical and structural studies indicate these residues contribute to transposase active site by coordinating the essential divalent cations (Mg^{2+} or Mn^{2+}).

Mu transposition occurs exclusively using a proper pair of recombination sites found at the ends of the phage genome. To address the mechanistic basis of this specificity, we have dissected the architecture of the tetrameric transposase. The four subunits in the tetramer can be distinguished as each is bound to a distinct DNA site on the ends of the Mu genome. By using recombination substrates in which the individual transposase binding sites are modified with IdU and ^{32}P, subunits bound to a specific site can be identified after UV-induced protein-DNA crosslinking. Using this method, we have determined both where an individual subunit binds on the DNA and where it catalyzes DNA joining. Our findings demonstrate that subunits do not catalyze recombination at the site adjacent to where they are bound, but rather on the opposite end of the phage genome. Thus, individual subunits bridge the DNA segments participating in recombination. Additional experiments indicate that subunits bound to two different sites contribute to catalysis of one reaction step. This intertwined subunit arrangement suggests a molecular explanation for the precision with which recombination occurs using a pair of DNA signals and provides an example of how the architecture of a protein-DNA complex can define the reaction products.

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A single active site catalyzes each of the chemical steps of Tn10/IS10 transposition in a sequential order.

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During Tn10 transposition, the element is excised from the donor site by double-strand cleavages at the two transposon ends, and the resulting 3'OH termini are then joined to target DNA. These chemical steps are catalyzed by the element-encoded (IS10) transposase protein, and occur within a synaptic complex containing the two transposon ends held together by monomers of transposase (Kleckner et al., 1995; Sakai et al., 1995). Cleavage of the two strands at each end occurs in a sequential order such that nicking of the transferred strand always precedes nicking of the nontransferred strand (Bolland and Kleckner, 1995). The single-strand nicked intermediate is seen to accumulate when Mn^{++} is substituted for Mg^{++} in the reaction or when certain mutant transposases are used, indicating that cleavage of each of the strands is a separate chemical event. For Tn10, double strand cleavage at both transposon ends is required prior to any association with target DNA (Sakai and Kleckner, personal communication), in contrast with other transposable elements like Tn7 or bacteriophage Mu that associate with target DNA before any cleavage has occurred (Bainton et al., 1993; Mizuuchi et al., 1992).

We have found four mutations in IS10 transposase, three in acidic residues, that individually abolish each of the chemical steps without affecting the synaptic complex (Bolland and Kleckner, 1996). Each of the catalysis-defective mutations blocks nicking of the transferred strand, nicking of the nontransferred strand on a substrate prenicked on the transferred strand, and strand transfer by a substrate precleaved on both strands. These results suggest that all three chemical steps in Tn10 transposition utilize a common set of critical amino acid residues. Furthermore, the analysis of reactions containing mixtures of wild-type and catalysis-defective transposases indicates that two monomers of transposase are sufficient to catalyze all four strand cleavages, implying that one active site cleaves both strands at one end. Also, the specific monomers that promote strand transfer have participated previously in DNA cleavage steps. The easiest interpretation of these results is that a single active site within a single monomer of IS10 transposase catalyzes cleavage of two DNA strands at a single transposon end and the subsequent joining of a single 3'OH end to target DNA. This interpretation differs from other models presented to explain transposition of bacteriophage Mu (Baker et al., 1994) and Tn7 (N. Craig, 1995), and thus points to the notion that even related transposable elements can exhibit alternative organizations of catalytic monomers within the synaptic complex.

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Single-Stranded DNA Intermediates in IS91 Rolling-Circle Transposition

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Transposable elements usually move by a mechanism which is called *transpositional recombination*. There is a first cleavage step, in which the transposon donor DNA is specifically cleaved at the two transposon ends to generate a pair of 3'-OH termini. There are two basic classes of transposons, depending if only a single strand is cleaved (Mu and retroviruses) or both strands are cleaved (Tn7 and Tn10) at the ends of the transposons. The second step, or strand transfer reaction, joins the donor 3'-OH ends to a target DNA (5).

IS91 was originally isolated from haemolytic plasmids of E.coli. It is 1830 bp long, contains dissimilar ends (called IRR and IRL), does not duplicate any sequence upon insertion, and shows an absolute requirement for target sequences CTTG or GTTC (1,2). Since its discovery, four other elements were found that are related to IS91: IS801, IS1294, ISFN1, and IS(WZU14952). We propose that IS91 transposes by a rolling circle mechanism, in a process that shares steps with the mechanism of replication of bacteriophage ϕX-174. The following properties of IS91 support this model:

1.- IS91 transposase belongs to the family of Rep proteins involved in rolling-circle replication of phages and plasmids (3). It acts efficiently in trans.

2.- IRR and the 5'-flanking sequence CTTG or GTTC are absolutely required for transposition, while IRL is dispensable. One-ended transposition of IS91 (in elements lacking IRL) occurs at similar frequencies than transposition of the complete element, and often results in tandem repetitions of the donor plasmid inserted in the recipient molecule. Any CTTG or GTTC sequence of the donor DNA molecule can be used as a secondary end (4).

3.- When expression of transposase is induced in vivo, ssDNA circles are produced by recircularization at the transposon termini. They are likely to be intermediates, or side-products, of IS91 transposition.

These properties of the IS91 transposon family are unique among transposable elements and possibly characterize a novel mechanism of transposition that we call rolling-circle transposition.

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Communications between Distant DNA Sites in Recombination by Resolvase

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During the transposition of Tn*3*-like elements, such as Tn*21*, the resolvase encoded by the transposon mediates a reciprocal recombination between two copies of the element, at their respective *res* sites. This site-specific recombination can be duplicated *in vitro* (Grindley, 1994). The addition of purified resolvase to a circular DNA with two *res* sites converts the DNA into two circles, interlinked to form a catenane. The only requirements for this reaction are that the DNA substrate is supercoiled and that the two *res* sites are in directly repeated orientation (Stark *et al.*, 1989). However, the overall reaction involves several stages. First, resolvase must bind to both *res* sites at their distant loci on the DNA (Avila *et al.*, 1990). Secondly, the two separate DNA-protein complexes must come together physically and interact with each other, to form the synaptic complex (Parker & Halford, 1991). Thirdly, both strands of the DNA at both *res* sites within the synaptic complex are cleaved and then re-joined to their opposite partners, to complete the strand transfer reaction (Stark *et al.*, 1992). The mechanism of resolvase thus includes one of the central processes in molecular biology, a communication event between two distant sites on a DNA molecule (Wang & Giaever, 1988; Schlief, 1992).

We shall describe here the application of rapid-reaction kinetic methods to analyse the mechanism of site-specific recombination by resolvase. The reactions of Tn*21* resolvase on a supercoiled plasmid with two Tn*21* *res* sites were monitored by three different assays. The three assays measured, respectively, either the initial binding of the protein to its target sites on DNA, or the synapse of the two DNA-protein complexes, or the final DNA exchange reaction. Binding was observed on a time scale from 5-100 milliseconds while the final reaction occupied 10-1000 seconds. Surprisingly, synapsis spanned this entire time range, with some DNA molecules yielding synaptic complexes within milliseconds after the initial binding while others took several hundred seconds. DNA synapsis during recombination by resolvase thus displays non-exponential kinetics.

Very few chemical reactions follow non-exponential kinetics because they can only arise from situations where each molecule of the substrate possesses a different reaction probability. In any normal reaction, the individual molecules all possess the same reaction probability, resulting in exponential kinetics. However, for DNA synapsis by resolvase, the individual molecules of the plasmid substrate will each possess a distinct conformation at the start of the reaction. Consequently, the motions of the DNA chain that lead to the juxtaposition of the two *res* sites will differ between each molecule. The non-exponential kinetics for the formation of synaptic complexes with resolvase may be the first direct analysis of the fluctuations in DNA structure that allow for communications between distant DNA sites.

The same spread of rates for synapsis were also observed on plasmids of different sizes and with different lengths of DNA between the sites. From polymer physics theory (Marko & Siggia, 1995), this excludes the possibility that distant sites on supercoiled DNA encounter each other by any process that requires the motion of the entire DNA chain. This contrasts to the behaviour of relaxed DNA, where the entire chain needs to move in order to juxtapose two sites that are far apart from each other. However, on supercoiled DNA, distant sites can encounter each other by a mechanism that involves the motion of only part of the chain, perhaps an individual branch within the supercoiled molecule.

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***In vivo and in vitro* studies on the circularization mechanism of the bacterial transposable element IS*911*.** **P.Polard, B. Hoang-Ton, L. Haren, B. Bétermier, R. Walczack and M. Chandler.** Laboratoire de Microbiologie et de Génétique Moléculaire du CNRS, 118 route de Narbonne, 31062 Toulouse cedex.

IS*911* transposase, called OrfAB, is produced by -1 programmed translational frameshifting between two overlapping reading frames, *orfA* and *orfB*. This type of expression couples the synthesis of the transposase with that of the product of *orfA*.. This protein, OrfA, appears to control the fate of the element displaced by the transposase. When both proteins are expressed, IS*911* is efficiently moved to a new locus, the intermolecular transposition reaction common to all transposable elements. However, if OrfAB alone is supplied, the reaction appears essentialy intramolecular, leading to the precise circularization of the element. The study of mutated elements *in vivo* indicates that this circularization process is a "half-transposition" event in which one end acts as a specific target for the transfer of a single donorend. This model has been reinforced by the characterisation of a second product generated during individual expression of OrfAB. This product is the result of the circularization of a single strand of the element without nicking of the second strand. Thus, if the donor molecule is a plasmid, it is converted into a "figure- eight" molecule. This is clearly a reflection of a one-ended transposition reaction. Kinetic and stability analysis has suggested that the eight form might be an intermediate in circle formation. To confirm this hypothesis and to determine the mechanism of circularization of the second strand, we have initiated *in vitro* studies. These have clearly indicated that formation of the eight molecule is catalysed by OrfAB in a two step reaction biochemically identical to that catalysed by retroviral integrases on proviral ends. This result is rather unsurprising because OrfAB shares with integrases the DDE motif, the catalytic site of such enzymes. The second strand circularization does not appear to be catalyzed by OrfAB. This will be discussed through the proposition of two alternative mechanisms, both involving the participation of specialized functions of the host.

POSTERS

Mobilization of Non-mobilizable Plasmids by The Aggregation-mediated Conjugation System of *Bacillus thuringiensis*

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In Gram-positive bacteria, four systems of DNA-transfer in liquid medium in which a bacterial clumping reaction leads to enhanced gene transfer have so far been described (6). One of these is the aggregation-mediated conjugation system in the mosquitocidal bacterium *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) (2). The mechanism of conjugation involves the formation of large aggregates of cells of different aggregation-phenotype (Agr^+ and Agr^-). Aggregation formation was found to be a prerequisite for successful conjugation. Jensen *et al.* (3) have shown that the conjugative apparatus and the genetic basis of the aggregation system is encoded by a 200 kb plasmid, pXO16, in *Bti*. We have "tagged" this aggregation-plasmid using the *B. thuringiensis* transposon *Tn*5401 containing a tetracycline-resistance gene and thereby monitored the host-range and kinetics of transfer to other *Bacilli*. Plasmid pXO16 is transferred to *Bti*-recipients with a frequency of approximately 100% during broth mating and severel *B. thuringiensis* subspecies and *B. cereus* strains were able to receive the plasmid and to express the aggregation and conjugation functions.

Mobilization of small non-conjugative plasmids by large conjugative plasmids or by conjugative transposons has been reported in some Gram-positive bacteria. Mobilization of plasmid pBC16 by the conjugative plasmid pLS20 in *Bacillus subtilis* is assumed to take place by a process analogous to mobilization by donation in Gram-negative bacteria (4). Mob proteins were found to be essential for the mobilization of pBC16 and pUB110 between *Bacillus* species in this conjugation system (4,5) The RS_A site of pUB110 has been shown to be involved in conjugative mobilization and it is suggested that the RS_A site may have a similar function as the Gram-negative *ori*T sites (5). We have used the aggregation-mediated conjugation system to examine the mobilization of various nonconjugative plasmids and their derivatives. Mobilization of the rolling-circle replicating plasmids pBC16 (mob^+,tet^R) and pAND006, containing the replicon of *Bti* plasmid pTX14-3 (mob^-,cam^R), were examined. Transconjugants appeared after a few minutes of mating and reached a maximum frequency after approximately two hours. Plasmid pBC16 was mobilized at a frequency of approximately 200 times that of pAND006. However, pAND006 was consistently transferred suggesting that the replicon of pTX14-3 is sufficient to sustain mobilization in *Bti*. Derivatives of pBC16 lacking the first part of the *mob*-gene and the RS_A site was also mobilizable in the *Bti* conjugation system at a reduced frequency, however. Other nonconjugative plasmids based on θ-replicating replicons were also mobilized consistantly in the *Bti* conjugation system.

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Genetic organization of the conjugal DNA metabolism region of the IncX plasmid R6K.

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We have analyzed the conjugal DNA processing region of the IncX plasmid R6K. Opposite to previously characterized conjugative plasmids, R6K contains two functional origins of transfer (*oriT*). The *oriT*s are formed by a common palindromic of 98 bp, invertedly orientated with respect to each other. They are located in the immediate vicinity of the α and β origins of replication. Insertional mutagenesis permitted us to identified the genes involved in the DNA conjugative metabolism: *taxA, taxB* and *taxC*. The genes were sequenced and compared with previously known *tra* genes of other systems. TaxA is homologous to TraY of plasmid F. Mutants in this gene significantly reduce the conjugative frequency (> 1000 fold). TaxB shows similarity with VirD4-like proteins. TaxC is the specific *oriT*-nickase, and belongs to the VirD2 nickase family. Thus, our characterization of TRA_X transfer confirms the commonality of the mechanism of conjugation in Gram-negative bacteria. Protein TaxC was overproduced and purified. It catalyzed *in vitro* a strand-transfer reaction on oligonucleotides containing its target sequence. The identified *nic* sites belong to the RP4/VirD2 *oriT* family. Cleavage was highly specific and did not occur with oligonucleotides containing *nic* sites of TraI of RP4 or VirD2 of the Ti plasmid despite their high similarity. Plasmid R6K DNA was isolated as relaxation complexes and specific nicks were induced by treatment with ethidium bromide. Nicked molecules were digested with snake venom phosphodiesterase to produce double strand cleavages at the *nic* sites. *nic* cleavage of *in vivo* pre-assembled relaxation complexes showed that at least 35 % of the molecules were cleaved simultaneously at both *nic* sites, and therefore this figure represents the minimum percentage of individual R6K molecules containing two pre-assembled relaxation complexes. This results suggest the necessity of a mechanism that controls the initiation of transfer from a single specific R6K *oriT*.

Genetic recombination events mediated by IS91 transposase

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IS91 inserts specifically at GTTC or CTTG target sequences without duplication of the target (1). IS91 termini play different roles in its transposition. The right inverted repeat (IR_R) and the flanking GTTC/CTTG target sequence are absolutely required for transposition. The left inverted repeat (IR_L) is dispensable. One-ended transposition of IS91 derivatives lacking IR_L, usually results in multiple tandem insertions of the donor plasmid in the target site (2). These results are easily explained by a rolling-circle replicative transposition mechanism. This model is also supported by the finding that the IS91 transposase is related in sequence to the superfamily of rolling-circle replication proteins and the observation that IR_R shows some conservation in sequence and secondary structure with the origins of replication of some rolling-circle replication plasmids (3).

In the present work we analyse the genetic characteristics of IS91 transposition such as cis-trans action of the transposase, one-ended transposition and intramolecular rearrangements mediated by IS91. We have also analysed IS91 native transposition products. Approximately the 87% of native transposition products are simple insertions of the element resulted from direct transposition. The remaining 13% were cointegrates and plasmid fusions; we assume these are the result of the failure in the IR_L recognitionwhen the transposase encounters IR_L during RCR. Fusions were obtained at about the same frequency as cointegrates. One-ended transposition occurred nearly at the same frequency ($2x10^{-4}$) as native element transposition ($9x10^{-4}$), and it resulted in multiple tandem insertions of the donor plasmid as it was previously described. In relation to its activity, IS91 transposase also acts in trans (transposition frequency:$3x10^{-4}$), whereas the rest of IS transposases only act in cis. We think that IS91 transposition might be regulated at the transcriptional level since transposition frequency was increased 100 fold after induction of transposase expression, which had been cloned under the control of the lac^{PO} promoter/operator. We detected loss of antibiotic resistance in donor plasmids which was the result of the insertion of a second IS91 element. We assumed these have been the product of intermolecular transposition since IS91 did not show immunity to transposition. There were plasmid deletions in some cases, although we consider they were the result of a secondary recombination between two copies of IS91. At the moment, we are analysing the products of IS91-mediated intramolecular rearragements: formation of excised transposon circles, plasmid deletions and inversions.

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Abstract:

"Analysis of a Region on the Conjugative Transposon Tc'DOT Which is Necessary and Sufficient for Transfer Activities"
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Members of the genus *Bacteroides* are gram negative, obligate anaerobes and constitute the numerically predominant genus in the human colon. Many *Bacteroides* clinical isolates harbor 80-200 kb conjugative transposons which excise from the chromosome of the donor, transfer via conjugation, and reintegrate into the chromosome of the recipient. Not only do these elements direct their own transfer, but they also act in trans to transfer coresident plasmids and to mediate the excision, circularization, and transfer of smaller, unlinked chromosomal elements called NBUs (Nonreplicating *Bacteroides* Units). It is believed that the transfer intermediate of the conjugative transposon is circular and that transfer occurs via a rolling circle model. Our lab has isolated a region within the conjugative transposon that is necessary and sufficient for transfer activities; plasmids into which this region is cloned are rendered self-transmissible. Sequence analysis of this region has unveiled at least 19 possible open reading frames, most of which had no sequence similarity with any known proteins. Only one open reading frame had significant similarity with known transfer genes, *traC* and *traB* from the *E. coli* F-plasmid, *traE* from the *Strep. agalactiae* pIP501, and *VirB5* from pTI of *Agrobacterium*. Some similarity was found between one open reading frame and the *mob* region of the NBUs, as well as several primase genes, but the significance of this similarity and the role of that open reading frame is unknown. The *oriT* of the conjugative transposon lies at one end of this region.

"A COUPLING ROLE FOR THE VIRD4 PROTEIN-FAMILY IN BACTERIAL CONJUGATION"

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Conjugation in Gram-negative bacteria is a DNA transfer process which occurs very efficiently via a multi-protein mating complex that assembles between two contacting bacteria. Two sets of proteins are known to be responsible for two specific steps in the process: PIL proteins, responsible for synthesis and assembly of the sex pilus and to produce the DNA transfer complex between donor and recipient bacteria (evolutionarily related to the VirB system, which allows transfer of the T-DNA from A.tumefaciens to plant cells) (Lessl and Lanka,1994) and MOB proteins that mobilize the DNA for conjugation and consist essentially of a nickase and accesory proteins, which form the relaxosome, and a third class of proteins the function of which is at present unknown and is the object of the present study: the VirD4-family. This group of proteins (TraD in plasmid F, TraG in RP4 and TrwB in R388) are homologous, and share this homology with VirD4 of the Ti-plasmid and MobB of CloDF13 (Cabezón et al.,1994).

The ability of conjugative plasmids from six different incompatibility groups to mobilize a set of mobilizable plasmids was examined. The mobilization frequencies of plasmids RSF1010, ColE1 and ColE3 varied over seven orders of magnitude, depending on the helper conjugative plasmid, while CloDF13 was always mobilized at high frequencies. It is shown that mobilization of CloDF13 is unique in that it does not require TrwB, TraG or TraD (all members of the VirD4 family) for mobilization by R388, RP4 or F respectively. CloDF13 codes for an essential mobilization protein (MobB) which is also a VirD4-homologue. Thus, mobilization of CloDF13 only requires the genes for pilus formation, suggesting that the TrwB-homolog is the protein which primarily determines the mobilization efficiency of a plasmid by its differential interaction with the various relaxosomes. To further test this hypothesis we analyzed the mobilization frequencies of ColE1 and RSF1010 by the P,W and F pilus in the presence of alternative TrwB-homologues. The results obtained suggest that the frequency of mobilization is determined both by the particular TrwB-like protein used and by the pilus system in a combined, but not linear fashion. Finally, the fact that TraG and TrwB are interchangeable for mobilization of RSF1010 and ColE1 by PILw but TraG cannot complement conjugation of trwB mutants suggested that specific interactions occur between TrwB and oriT(R388) that are not essential for mobilization. Thus, we searched for mutations in trwB which affected self-transfer but not mobilization using a series of trwB mutants. No mutants were found that impaired self-transfer but were not affected in mobilization. Therefore, we favor the alternative possibility that mobilization and self-transfer use TrwB in the same way, and the differential interactions of TrwB with pilus and relaxosome are the factors that determine the frequencies of transfer of different MOB regions.

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The role of the chromatin-associated protein Hbsu in β recombinase-mediated DNA recombination is to facilitate the joining of distant recombination sites

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The β recombinase, encoded by the *Streptococcus pyogenes* plasmid pSM19035, is involved both in the resolution of plasmid multimers into monomers and in DNA inversion. The β recombinase binding site has been localized to an 90-bp region (crossing over site, *six* site) that can be divided into two discrete subsites (I and II). These subsites, which are about 34-bp in length, are separated from each other by about 16-bp. Subsite I contains two 10-bp imperfectly conserved sequences with dyad axis symmetry, whereas in subsite II such a dyad axis of symmetry is not evident. The highly purified β recombinase is unable to mediate *in vitro* DNA recombination unless a chromatin associated protein such as *Bacillus subtilis* Hbsu or *Escherichia coli* HU is provided. In the presence of Hbsu or HU, and of a supercoiled DNA substrate, DNA resolution is obtained when the two *six* sites are directly oriented, whereas DNA inversion is the product when the *six* sites are in inverse orientation. Electron microscopy has provided direct evidence that the role of the chromatin-associated Hbsu protein is to promote the assembly of the distant recombination sites. The β protein can bind to DNA in the absence of Hbsu. The mammalian chromatin-associated protein HMG1, sharing neither sequence nor structural homology with Hbsu or HU, also stimulates β-mediated recombination. Furthermore, in the presence of the β protein and of a supercoiled DNA substrate, Hbsu seems to have a marked binding preference for an unknown site in the *six* region. Considering that the Hu, Hbsu and HMG1 proteins bind preferently to bent or altered DNA structures, we propose that Hbsu facilitates the assembly of the synaptic complex by stabilizing a bent or altered DNA structure in the recombination complex.

The DNA transport complex encoded by conjugative IncP plasmids functions as bacteriophage PRD1 receptor

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Conjugative DNA transfer requires functions for mating pair formation (Mpf) as well as for DNA processing and transport. The Mpf complex is associated with the cell envelope and is believed to function in several processes. During bacterial conjugation it is involved in contact formation between the donor and the recipient cell, and it functions as a DNA transport structure. It is also used by male specific bacteriophages as the phage receptor and the DNA entry site into the cell.

Functions responsible for conjugative transfer of plasmid RP4 (IncP) are encoded by two distinct regions, Tra1 and Tra2. The Tra1 and Tra2 core regions encoding essential transfer functions have been identified and evaluated by genetic analyses[1,2,3]. One gene product from Tra1 (TraF) and eleven Tra2 core gene products (TrbB,C,D,E,F,G,H,I,J,K,L) form the Mpf complex. Most of the Trb proteins are likely to locate to the bacterial membranes as indicated by their amino acid sequences[4]. Each of the Tra2 core region genes except for *trbK* (*eex*, entry exclusion[8]) are essential for RP4 specific plasmid transfer. We are further characterizing the structure and function of the Mpf complex using biochemical approaches and electrochemical and genetic methods.

Bacteriophage PRD1 is a lipid-containing dsDNA virus[5], which uses the IncP-encoded Mpf structure as a receptor[6,7]. The same functions needed for RP4 transfer are involved in PRD1 propagation[8]. We are carrying out mutational analysis of the Mpf complex proteins by isolating spontaneous PRD1 resistant mutants. 312 PRD1 resistant mutants affecting Tra2 genes have been isolated. None of these mutants adsorbed the phage, thus not a single mutant defective in later steps in infection was obtained. Mutations are distributed within the Tra2 core region and accumulated to two genes, *trbL* and *trbE*. The correlation between phage resistance and conjugative transfer was analyzed by testing the transfer proficiency of the phage resistant mutants. Seven out of the 312 mutants were transfer positive, but showed reduced transfer frequencies compared to wild-type RP4 frequency. These mutations map to four different *trb* genes (*trbG,I,J,L*).

During conjugative transfer a DNA-protein complex is thought to be transmitted through a channel or pore at the mating bridge between the donor and the recipient cells. We have shown using electrochemical measurements that the Mpf complex, in the absence of DNA transfer, makes the cells permeable to lipophilic compounds and increases membrane voltage[9]. Similar effects are induced by PRD1 DNA entry into the cell as shown in permeability measurements with PRD1 infected cells[10].

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Characterization of domains of the replication protein RepX of the rolling circle plasmid pFX2

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Rolling circle replicating plasmids, constituting a group of small promiscuous multicopy replicons, can be divided into 4 subfamilies, according to their double strand origins (Novick, 1989). One of these consists of the plasmids pLS1, pE194 and pFX2, whose replication initiator proteins share significant homologies especially at their respective N-terminal ends. del Solar et al. (1993) proposed that at least two domains must exist in the Rep proteins of the pLS1 family: i) one conserved domain located at the N-terminal moiety, supposed to be involved in the nicking reaction and ii) a second, not conserved (in the core region), probably involved in the binding to the iterons.

The coding region for the Rep protein of the lactococcal plasmid pFX2, RepX, was inserted into the glutathione S-transferase (GST) fusion vector pGEX-2T and expressed by induction of the *tac*-promoter with 0.1mM IPTG. After 30 minutes of induction, expression of the ca. 52kDa fusion protein was detected, the expression level (*E. coli* JM109 as host strain) increased continuously and reached a maximum after 4 hours of induction.

Single amino acid changes in three different domains of the RepX protein of pFX2 were performed by *in vitro* site-directed mutagenesis and recombinant PCR technology (Higuchi et al., 1988), respectively. These show homologies to the respective regions in the Rep proteins of the pLS1 family, namely to the putative metal-binding domain at the N-terminal moiety as well as to two conserved blocks in the core region, thought to be involved in the iteron-binding activity. The mutant RepX proteins were expressed in the same way as glutathione S-transferase fusion proteins and purified via affinity chromatography.

Results of *in vitro* replication assays employing the purified RepX proteins (original and mutagenized) and supercoiled plasmid DNA of pFX2, pLS1 and pE194 in the presence of different divalent metallic ions, will be shown.

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System to study horizontal gene transfer by conjugation

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In order to study horizontal gene transfer by conjugation in the environment it would be valuable to have a system that provides stably inherited traits in a broad range of bacteria . Plasmid incompatibility and plasmid replication in a restricted range of bacteria are the limiting factors in using plasmid vehicles to study horizontal gene transfer. To exclude these problems, we have designed a genetic system which allows the integration of marker genes into the highly conserved ribosomal RNA genes as sites for homologous recombination after DNA uptake. Because of the ribosomal RNA operon multiplicity in most procaryotic chromosomes, the inactivation of one of these *rrn* operons has no observable influence on cell growth or physiology.

Conjugative plasmids, containing 16S ribosomal RNA gene sequences from different unrelated bacteria, followed by the *aphA-3* kanamycin resistance gene, were constructed. The origin of replication of the narrow-range plasmid ColE1 was used in order to prevent the replication of the plasmid in the recipients. In conjugation experiments, using *E. coli* as donor and different recipient bacteria, the plasmid transfer resulted in the integration of the plasmid into the chromosome by homologous recombination between the 16 S rRNA gene sequences. The recombination event could be detected by Southern blot analysis, or by PCR amplification of the recombinant sequence using a universal primer for the 16S rRNA gene upstream of the recombination site and a reverse primer specific for the *aphA-3* marker gene (Fig. 1). This PCR amplification enables the detection of gene transfer in nature without cultivation of the recipient cells and sequencing of the PCR-amplified 16S rDNA provides a phylogenetic assignment of the recombinant bacteria.

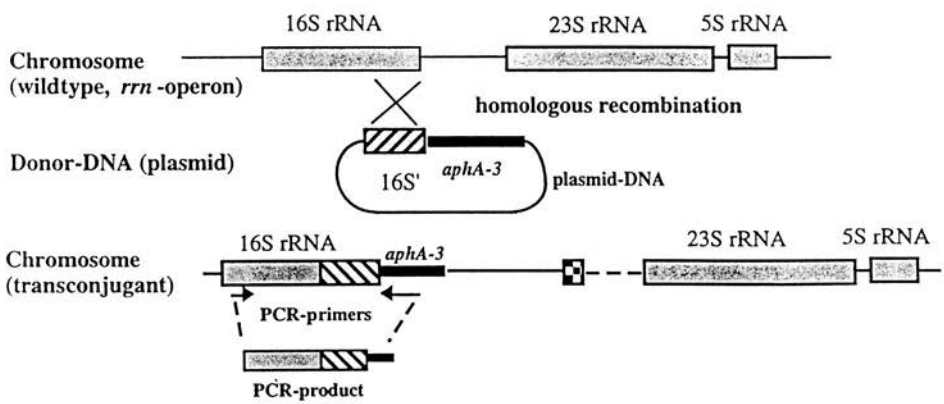

Fig. 1: Scheme for gene integration between plasmid DNA and chromosome

PROTEIN-DNA INTERACTIONS AT THE oriT OF PLASMID pMV158

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Transfer of plasmids by conjugative processes is a widespread mechanism which has been well characterized in replicons isolated from gram-negative bacteria (1). Plasmid-mediated DNA transfer requires two conserved elements: i) a *cis* element where the transfer process starts (*oriT*), and ii) a site-specific endonuclease that cleaves one strand of the *oriT* at a particular site, generally termed *nic* site. The group of plasmids replicating by the rolling circle (RC) mode (2, 3, 4) may encode a protein, generically termed Mob, supposedly involved in their mobilization by other plasmids (5, 6). So far, little information is available on these Mob proteins and on their interactions with the plasmid *oriT* DNA. Based upon similarities with well characterized transfer mechanisms, it is believed that Mob proteins from RC-replicating plasmids would introduce a nick at the *oriT* (1). Mob proteins share structural and functional similarities (7) with the plasmid-encoded proteins (termed Rep) involved in the initiation of rolling circle replication, both kind of proteins nicking their cognate origin DNA (*dso* for Rep proteins, *oriT* for Mob proteins).

The streptococcal plasmid pMV158 (5536 bp) has been shown to be mobilized by plasmids of the pIP501/pAMβ1 family (5, 6). pMV158 contains a *mob* gene (which we have termed *mobM*) which is required for plasmid mobilization, and which could encode a protein of 58 kDa, MobM. Genes highly homologous to the pMV158-*mobM* have been reported for some staphylococcal plasmids (like pUB110) replicating by the RC mode (5, 6).

We have identified relaxation complexes, due to the presence of an intact *mobM* gene, in *Streptococcus pneumoniae* harbouring pMV158. The gene has been cloned in an expression vector, and the MobM protein has been overproduced, purified, and the sequence at its N-terminal end has been determined. We have shown that purified MobM protein specifically cleave supercoiled pMV158 DNA (forms FI), generating open circular forms (forms FII). Relaxed molecules (forms FI'), derived from a nicking-closing reaction have not been found. Some parameters of the nicking reaction

have been determined. Nicking requires the presence of Mg^{2+} or Mn^{2+} ions in a temperature-dependent reaction. Time-course and concentration-dependence reactions showed that generation of forms FII quickly increased with time, reaching about 50% after 30 min of incubation at 30°C.

MobM-DNA complexes have been isolated after precipitacion of reaction products with KCl in the presence of SDS, indicating the existence of a covalent linkage of MobM with its DNA target. The nick introduced by MobM on supercoiled pMV158 DNA has been identified by primer extension assays and by determination of the DNA sequence of FII molecules.

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TrbK, a small hydrophilic cytoplasmic membrane lipoprotein, functions in entry exclusion of the IncPα plasmid RP4

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Introduction

It is known for many conjugative plasmids, that DNA transfer rates are reduced if the recipient already contains an identical or a closely related plasmid as the donor. The phenomenon is called entry or surface exclusion. For F and F-like plasmids two genes, *traT* and *traS*, have been identified to contribute independently to the Eex-phenotype (2,6). The IncI plasmid R144 also encodes two entry exclusion proteins (4,5). Other plasmids like the IncN-plasmid pKM101 encode only one gene product with an entry exclusion function (8). TraT of F is thought to prevent the formation of stable mating pairs (1). However, little is known about the mechanism, by which Eex proteins inhibit the entry of conjugatively transferred plasmid DNA into the recipient cell.

The entry exclusion function of RP4

A single gene, *trbK*, which maps in the Tra2 region was found to be responsible for the entry exclusion function of RP4 (3). TrbK is a small hydrophilic protein of 69 amino acids, that contains a lipoprotein signature (7). It is inessential for DNA transfer. The *trbK* gene has been cloned separately and was shown to function independently of other Tra2 genes (3). Transfer frequencies are also reduced in matings between recipient cells containing TrbK and donor cells that lack TrbK, indicating that for the entry exclusion function the protein acts only at the recipient's side.

Entry exclusion mediated by TrbK is specific for IncP plasmids. The plasmids IncPα R702 and RP4 were excluded by TrbK to the same extent. The transfer of the IncPβ plasmid R751 is also affected by TrbK. However, the Eex-index for R751 was about three times lower than for the IncPα plasmids studied. This indicates that entry exclusion by TrbK depends on the degree of relationship between two plasmids.

Processing and localization of TrbK

Overexpression of *trbK* using the P_{tac}-*lacI*^{q}-system results in the appearance of two bands in a Coomassie-stained tricine-SDS polyacrylamide gel, corresponding to approximately 5.5 and 7kDa. This suggests that the smaller protein represents the mature polypeptide after lipid modification and subsequent proteolytic cleavage by the lipoprotein peptidase. Both, the modified and nonprocessed form of TrbK, were found to localize predominantly to the cytoplasmic membrane in *E.coli*. About 15% of the protein was found in the outer membrane fractions. We therefore assume that TrbK acts at the periplasmic side of the inner membrane.

Mutational analysis of *trbK*

To further investigate the structure and function of TrbK, two mutants were created by site-directed mutagenesis. First, the lipid attachment site cystein-23 was changed to glycine (*trbK*C23G) to impair lipid modification and hence processing of the protein precursor. The second mutation (*trbK*K62⋆) changed lysine-62 to an amber codon leading to a C-terminal truncation of eight amino acids. SDS cell extracts of the two mutant strains were analyzed by gel electrophoresis and subsequent Coomassie-staining or solid phase immunoassay. The C23G mutant lacks the processed form of about 5.5kDa, indicating that the protein cannot be modified like the wild-type protein. In extracts of the K62⋆ mutant two bands of about 4.5 and 6kDa were identified, indicating C-terminal truncation as well as normal processing at the N-terminus of the protein.

Membrane distribution of the TrbK mutant proteins was not altered compared to the wild-type protein, suggesting that neither the lipid modification and processing nor the C-terminal eight amino acids apparently influenced the correct localization of the protein within the cell envelope. However, incorporation of the mutant protein TrbKC23G into the membrane seemed to be somewhat inefficient or weaker since we detected only this protein in the cultural supernatant and in higher amounts in the soluble fractions as compared to the wild-type and the K62⋆ mutant. The entry exclusion capacity of the TrbK mutant C23G was found to be reduced about 10-fold compared to the wild-type. However, no entry exclusion ability was detected with mutant K62⋆, indicating that the C-terminal amino acids are essential for TrbK function.

Interaction of TrbK with the RP4 Mpf-system

To answer the question which component(s) of the RP4 transport machinery is recognized and affected by TrbK, we studied the influence of TrbK on mobilization of RSF1010. We found that mobilization of RSF1010 by the RP4 Mpf-system to a recipient that contains TrbK is decreased to the same extend as the mobilization of the RP4 Tra1 region. This indicates that TrbK specifically recognize one or more components of the Mpf-system of RP4, which is needed for both, RP4 transfer and RSF1010 mobilization. TrbK might interact directly or indirectly with the transport structure, inhibiting a critical step of the DNA transfer, probably after the cell-to-cell contact has been established.

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The mating pair formation system of the IncPα plasmid RP4: Functional and structural aspects

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Introduction

The self-transmissible IncPα plasmid RP4 (60,099 bp) (7) is known for its extremely broad host range, i.e. it is stably maintained in all tested Gram-negative bacteria. Furthermore, it can be transferred by conjugation to a variety of different organisms such as Gram-positive bacteria and even yeast (2,8). Therefore, RP4 serves as a widely used model system for the study of plasmid biology in general and conjugative DNA transfer between a donor bacterium and a recipient cell. In addition, several findings provide evidence for a close relationship of RP4-mediated bacterial conjugation and T-DNA transfer from agrobacteria to plant cells. It has been shown that the gene organization and amino acid sequence of the gene products of the RP4 Tra regions and the Vir regions of Ti plasmids of *Agrobacterium tumefaciens* express a remarkable degree of similarity (3,6).

Two distinct regions of RP4, Tra1 and Tra2, encode essential transfer functions. The Tra1 region mainly encodes DNA processing functions for generating and release of the single-stranded DNA molecule, which is transferred to the recipient. The Tra2 core region (11 genes) and *traF* of Tra1 belong to the so-called mating pair formation (Mpf) system, which is required to establish an intimate cell-to-cell contact between donor and recipient cell. In addition, the gene products of the Mpf-system are thought to form a proposed transport structure for transmission of DNA through the membranes into the recipient cell (1,4).

From our studies we postulate three major components, which are required for conjugative transfer of RP4 into the recipient cell: (i) the relaxosome, which is formed by the gene products of *traH*, *traI*, *traJ*, and *traK* encoded by the Tra1 region at the transfer origin (*oriT*). (ii) the Mpf-system and (iii) TraG, which is a Tra1 component and is thought to connect the relaxosome and the Mpf-complex. After the intimate cell-to-cell contact between the donor and the recipient has been established by the Mpf-gene products, a not yet defined signal is assumed to trigger the release of the single-stranded DNA molecule from the relaxosomal complex, which initiates the DNA transport into the recipient.

The RP4 Mating pair formation system

The RP4 Mpf-system is a member of a superfamily of related export systems for macromolecules. These include the T-DNA transfer system of Ti-plasmids, e.g. the VirB operon, conjugative transfer systems of other plasmids like the Tra regions of the IncN plasmid pKM101 and the IncW plasmid R388, as well as the Tra3 region of the Ti-plasmid (see abstract of S. Farrand). Most remarkably the Mpf system of RP4 is also related to the pertussis toxin export system, the Ptl operon, of *Bordetella pertussis*. The transport structures and the mechanism of the macromolecule export process have not been studied extensively in any of these systems (for review see ref. 5).

The essential components of the Mpf system were determined using defined insertion mutations in each gene of the Tra2 core region. Ten Tra2 genes, *trbB*, *trbC*, *trbD*, *trbE*, *trbF*, *trbG*, *trbH*, *trbI*, *trbJ*, and *trbL*, but not *trbK* were found to be essential for RP4-specific transfer. Several criteria serve to further characterize the RP4 Mpf system: (i) Mobilization of the non-self-transmissible IncQ plasmid RSF1010. RSF1010 encodes its own relaxosomal components, but its transfer to a recipient cell relies on the Mpf systems of conjugative

plasmids; (ii) Propagation of donor-specific phages (PRD1, Pf3, PRR1) and (iii) production of extracellular filamentous structures (pili). The same components, which are essential for RP4 transfer, are also required for RSF1010 mobilization, for pilus assembly, and for the formation of the phage receptor (see abstract of A. M. Grahn *et al.*). The only gene, which is not an essential RP4 Mpf component, is the entry exclusion gene, *trbK* (1).

Localization of the components of the RP4 transfer apparatus

As mentioned above, the RP4 Mpf system is thought to encode a membrane structure, which is responsible for the establishment of the intimate cell-to-cell contact. This complex facilitates the DNA transfer into the recipient and serves also as a receptor complex for donor-specific phages like PRD1. Little is known about the architecture of the RP4-encoded or other related membrane complexes (see above). Signal peptide processing was observed for some Tra2 gene products (1,4). In addition, we found transmembrane helical, other hydrophobic and amphiphilic segments within many of the Mpf components and also in some of the Tra1 proteins including TraF and TraG. These findings suggest a localization in or an association with the bacterial cell envelope. To determine the cellular localization of RP4 transfer proteins we used specific polyclonal antisera raised against purified transfer gene products.

Most of the Tra2 gene products, except TrbB and TrbC, localized to both membrane fractions, cytoplasmic and outer membrane. However, the majority of these proteins were most abundant in the outer membrane fraction. TrbB was detected in the soluble and the cytoplasmic membrane fractions. This protein is thought to function as a so-called *traffic* ATPase. TrbC localizes to the outer membrane. However, this protein was not detectable in membrane fractions of cells, that contained TraF or the whole of the Tra1 region in addition to Tra2. Two other proteins of the Tra2 region, TrbG and TrbJ, were also detected in the soluble fractions representing the cytoplasm and the periplasm, as well as in both membrane fractions. Since signal peptide cleavage was observed for these proteins. It is suggested that they are localized in the periplasm interacting directly or indirectly via protein-protein-interactions with the cytoplasmic and the outer membrane.

The Tra1 component of the Mpf system, TraF, and the "connecting" protein TraG were also detected in the membrane fraction. This localization supports the idea that they function in mating pair formation (TraF) and in connecting the relaxosome and the Mpf complex (TraG). TraI, the relaxase, was most abundant in the cytoplasmic membrane, but the protein was also detected in the soluble fraction. According to their mainly hydrophilic character, the remaining Tra1-encoded gene products are found in the soluble fraction, i.e. the cytoplasm. However, almost all of these proteins, with the exception of TraJ, were also weakly detected in the cytoplasmic membrane. Direct interaction with the membrane via hydrophobic or amphiphilic segments or indirect association via protein-protein-interactions might be assumed.

Conclusion

The observed localization of the different components of the transfer complex support our model of the mechanism by which the conjugative DNA transfer is mediated. The Mpf components, encoded by the Tra2 region and *traF*, form a membrane channel or pore spanning the cytoplasmic and outer membrane. Components of the relaxosome were found associated with the cytoplasmic membrane. Thus this complex might be assembled directly in the cytoplasmic membrane. It is also possible that the relaxosome is assembled in the cytoplasm and is then hooked up to the membrane. TraG is neither a component of the relaxosome, nor the Mpf-complex. However *traG* is, in addition to Mpf, essential for mobilization of the non-self-transmissible IncQ plasmid RSF1010. Thus, TraG is the most likely candidate to functionally link the two complexes by interacting with both, the

relaxosome and Mpf, facilitating the release of the single-stranded DNA molecule and its transport into the recipient after the close cell-to-cell contact has been established by the Mpf-system.

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THE HHA/YMOA FAMILY OF PROTEINS: MODULATION OF BACTERIAL GENE EXPRESSION IN RESPONSE TO ENVIRONMENTAL STIMULI

Proteins belonging to the Hha/YmoA family have been found to participate in the modulation of the expression of enterobacterial virulence factors in response to environmental factors. The Hha protein has been identified in *E. coli* as being a modulator of the expression of the toxin α-hemolysin. Synthesis of this toxin is repressed both at high osmolarity and at low temperature. *Hha* mutants derepress hemolysin expression when cells grow either at low temperature or in a high osmolarity medium. *ymoA* mutants were isolated in *Yersinia enterocolitica* as expressing at low temperature different virulence factors (the Yop proteins and the YadA adhesin) that are otherwise repressed when cells grow at about 20-22° C. Both proteins share extensive homology in their aminoacid sequence and, being the phenotype of *hha/ymoA* similar to that of *hns* mutants, it was suggested these proteins being histone-like proteins. In fact, the degree of plasmid DNA supercoiling is altered in *hha* mutants and factors modifying the degree of DNA supercoiling such as high osmolarity and gyrase inhibitors mimic the effect of the *hha* allele increasing the expression of the *E. coli* toxin α-hemolysin encoded by the recombinant plasmid pANN202-312

In addition to modulate expression of different genes in response to environmental stimuli, these proteins appear to participate in other cellular processes. Thus, subculturing of many Tn*5*-induced *hha* mutants renders *hha*$^+$ cells that contain Tn*5* inserted in other locations. It has been suggested that the lack of a functional Hha protein is responsible for the excision of Tn*5* from the *hha* gene. Related to this, it has been recently shown that *E. coli* cells carrying the *hha* gene cloned in a medium-copy-number plasmid (15-20 copies per cell) present a high frequency of IS transpositions. This effect, that is not observed when the gene is cloned in a low-copy-number plasmid (1-5 copies per cell), clearly indicates an effect of the overexpression of the *hha* gene on IS mobilization from the *E. coli* genome and could also suggest that Hha may have a role on stability of transposable elements in the *E. coli* genome. This point is currently being investigated.

A recent comparison of the amino acid sequence of the Hha protein to the non-redundant peptide sequence database of the Blast network service showed a very high homology of Hha to a previously unnoticed open reading frame (ORF) corresponding to the promoter-distal genes of the *tra* operon of plasmid R100. We have tentatively named RmoA to the gene product encoded by such ORF and are currently investigating if, in fact, conjugative plasmids such as R100 code for such protein and, if it is the case, its role in the conjugation process.

The Role of TrwA Protein on IncW Plasmid R388 Conjugation

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Bacterial conjugation is a process whereby plasmid DNA in donor cells is transferred to recipient cells by a mechanism which requires cell to cell contact. This process is initiated by the cleavage of a phosphodiester bond at a particular site of the origin of transfer (*oriT*) by a site-specific endonuclease that allows the creation of a single strand by subsequent strand displacement through rolling circle-type replication.The single strand is then transferred into the recipient cell and its complementary strand is synthesised to form an intact plasmid. In addition to the site specific endonuclease, conjugative plasmids need other *oriT* binding proteins involved in the enhancement of the nicking reaction or in other functions as DNA membrane anchor, initiation signal transmission or induction of topological changes.

In IncW plasmid R388, three proteins are involved in DNA processing for conjugal transfer, TrwA, TrwB and TrwC. TrwC protein has shown *in vitro* DNA helicase activity and *oriT*-specific endonuclease activity, remaining covalently associated to the nicked strand 5' end. TrwB amino acid sequence predicts an integral membrane protein with a putative NTP binding motif that belongs to the VirD4-protein family.

In order to understand the role of TrwA in the initiation of conjugal DNA transfer of plasmid R388 we have purified the protein from IPTG induced *E. Coli* cells.The N-terminal amino acid sequence of the purified TrwA protein was determined to be identical to the sequence predicted from the nucleotide sequence of the R388 TrwA gene with the exception of the initial Methionine. To address the question whether native TrwA protein exists on an oligomeric form in solution, cross-linking studies were performed using glutaraldehyde and they showed that TrwA protein forms predominantly a tetramer in solution, with a molecular mass of 53,084.This result was consistent with gel-filtration data, where TrwA eluted as a protein of, aproximately, 55 KDa.

Gel shift assays showed that TrwA protein binds specifically to the *oriT* sequence. We performed DNAse I footprinting analysis to precisely define the TrwA protein binding sites.Two DNA regions within the *oriT* region (regions A and B) were protected by TrwA. At low TrwA concentrations ($<2x10^{-7}$ M) only region A was protected while region B required higher TrwA concentrations. Gel shift assays with fragments of *oriT* containig various deletions also demostrated that the binding to B is dependent to a previous binding to A.

These *in vitro* results can be correlated with *in vivo* data using deletions in *oriT*. For example, deletions in the A binding site result in a 1000-fold decrease in transfer frequency, the same decrease produced by the lack of TrwA. Deletions into the B site had no further effects in the frequency of transfer, suggesting that binding to B is also dependent *in vivo* to a previous binding to A.

When *oriT*-nicking assays by TrwC were carried out in the presence of TrwA no variation could be detected, so we assume that TrwA is not involved in *oriT*-nicking. This fact, together with the DNA-binding data showed above, suggested a simmilar function to TraM of plasmids R100 and F or TraK of RP4. These proteins have been implicated in functions as DNA membrane anchor, initiation signal transmission or induction of topological changes.

TrwD: An ATPase involved in the conjugative process of plasmid R388

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Bacterial conjugation is a DNA-transport process that takes place by a multienzimatic mechanism with oustanding efficiency. Analysis of the DNA sequence of plasmid R388 showed 4 genes encoding proteins that contain NTP-binding sequences (one of those genes is *trwD*). The function of these proteins could be to provide the necessary energy to the DNA transfer apparatus or, alternately, to act as mediators of the mating signal.

TrwD is an essential protein in the conjugative transfer of plasmid R388, as shown by genetic complementation analysis. *trwD* was cloned as a fusion with the gene of the glutathion-S-transferase (GST) to get an easier purification of the fusion protein. GST-TrwD was able to complement *trwD* mutants with similar transfer frequencies to those found for R388 wild type plasmid.

Fusion protein was purified from overproducing bacteria. Factor X_a hydrolysis of GST-TrwD and further purification rendered TrwD protein with more than 95% purity.

ATP hydrolysis was observed, both by purified GST-TrwD and TrwD, with a Km of about 0.16 mM and an optimum pH of 9.0. ATPase activity required Mg^{2+} and showed a sigmoidal increase with protein concentration, suggesting that ATPase activity requires a multimer of TrwD. Preliminary results suggest that TrwD forms hexamers in solution. The weak ATPase activity of TrwD (1-5 nmol ATP/mg/min at 30°C) was increased 3-fold in the presence of small unilamellar vesicles of PC:PL (1:1) and PC:PG (1:1). No stimulation of the ATPase activity occurred with ssDNA, dsDNA, micelles of SDS, TX-100 or lysoPC, or sodium sulphite. ATP hydrolysis was not reduced by comon ATPase inhibitors such DCCD, thapsigargine, oligomycine or sodium orthovanadate.

In order to study the importance of the nucletide-binding site Walker box A (GxxGxGKT/S) present in TrwD, the conserved lisine residue was replaced by glutamine. The mutant protein, expressed and purified at the wild-type conditions, did not exhibit ATPase activity. The GST-TrwD(K432Q) was not able to complement the mutation in *trwD* of the R388 mutant plasmid, suggesting the essentiality of the ATPase activity of the protein in the conjugative process. Furthermore, when the donor strain contained both R388 plasmid and the mutant plasmid, the conjugation frequence decreased one thousand fold with respect to the wild type R388. This fact indicated the dominant character of this mutation, suggesting that GST-TrwDK432Q is still able to interact with another component of the conjugative machinery.

Antibodies raised against TrwD localized it in the inner membrane of *E. coli*. TrwD is probably a peripheral inner membrane protein since it could be solubilized by increasing salt concentration to 0.5 M NaCl.

Our work at the moment is centered on the search of the biological function of TrwD. We are trying to further characterize the ATPase activity of the protein and studying potential protein-protein interactions in which TrwD is implicated.

Functional analysis of TraM, a DNA binding protein essential for conjugative DNA transfer of F-like plasmids.

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Protein TraM has an essential function in the conjugation process of IncF plasmids like F, R1 or the enterotoxin plasmid P307. We have previously shown in the R1-system, that TraM is a DNA-binding protein, attaching to two closely spaced regions between the origin of transfer (*oriT*) and its own structural gene (1). Since this region contains the two traM-promoters we suggested that TraM acts as an autoregulatory protein. This proposition was recently experimentally documented (2). The N-terminal part of TraM is predicted to form an amphiphilic α-helix which seems to be the primary DNA-binding domain, as we could show by introducing mutations in this part of the molecule. The amphiphilic helix also confers specificity to the autoregulatory capacity of TraM. A truncated TraM protein with the C-terminal half of the molecule deleted shows some residual autoregulatory activity underscoring the principal importance of the N-terminal region. However, this result also indicates that the C-terminal domain is necessary for proper autoregulation which presumably requires tetramerization of the protein. Three other point mutations within the internal domain of TraM retained their autoregulatory activity.

Besides testing the created mutants for autoregulation, we also scrutinized their effects on the conjugative activity of a R1 plasmid derivative created by an allelic exchange method. We thereby created several isogenic conjugative plasmids producing altered TraM molecules. The results of these studies and studies with binding site mutants will be presented. They surprisingly revealed that TraM plays a key role in the complex regulatory circuitry controlling transfer gene expression via a long distance effect, possibly through altering DNA topology.

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Workshop on

ENZYMOLOGY OF DNA-STRAND TRANSFER MECHANISMS

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