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Translocation of DNA across Bacterial Membranes

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INTRODUCTION

DNA translocation across bacterial membranes occurs at the early stages of several important biological processes such as infection by bacteriophages, conjugative DNA transfer, genetic transformation, and T-DNA transfer from bacteria to plant cells. During these processes the DNA traverses the cell envelope including the cytoplasmic membrane, the periplasmic space, and the outer membrane of bacteria. The passage of DNA across the bacterial envelope poses several problems. (i) The hydrophobic bacterial cytoplasmic membrane is a barrier for macromolecules and thus also for DNA. (ii) The outer membrane of gram-negative bacteria is negatively charged (mainly as a result of the lipopolysaccharide content) and thus is a hindrance for translocation of molecules like DNA with a high-density negative charge. (iii) Nucleases present in the periplasmic space may attack the DNA during transfer. Thus, to translocate DNA across bacterial membranes, specific DNA translocation devices have evolved. Meanwhile, a considerable number of studies on the processes and structures involved in DNA translocations have been performed, and it is the scope of this review to summarize data, interpretations, and also some speculations which have led to the current models for DNA translocations. The biological phenomena which greatly depend on DNA translocation processes have been reviewed regularly over the years in their microbiological, genetic, physiological and ecological aspects (see below). However, the mechanism of DNA translocation across the membrane itself

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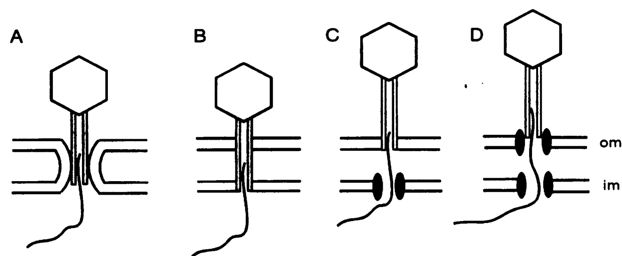

FIG. 1. Models for DNA passage during infection of cells by bacteriophages. (A) Preexisting or induced membrane fusions allow DNA translocation without crossing of membranes (e.g., T4). (B) Passage of DNA through channel-forming phage tail proteins spanning all envelope layers of gram-negative bacteria (e.g., T5). (C) The pore in the outer membrane (om) is provided by the phage; the pore in the inner membrane (im) is provided by the host (e.g., fd). (D) Different channels in the outer and inner membranes, both formed of host proteins, mediate DNA passage (e.g., lambda). In models C and D, DNA is translocated across the periplasm.

was not addressed in detail in most of these reviews, although there were a few articles comparing the structural basis (160, 247, 264) and the energetics (113, 217a) of DNA transfer in different systems. Because the bioenergetic aspects of DNA translocation have been treated in a recent review (217a), they are omitted here.

The increasing general interest in DNA membrane translocation seems to derive its stimulus from several developments in the recent exploration of prokaryotic biology. Besides the natural processes of DNA uptake, the experimental manipulation of these processes and of cells allowing transformation is a very important tool in genetic engineering. In this context, risk assessment studies on the release of genetically engineered microorganisms with emphasis on horizontal gene transfer have led to a broad conception of gene transfer among prokaryotes and even prokaryotes and eukaryotes, culminating in a term like "transkingdom sex."

TRANSLOCATION OF PHAGE NUCLEIC ACID ACROSS THE ENVELOPE OF THE HOST CELL

Numerous studies have been performed to elucidate the mechanism by which phage nucleic acid enters a bacterial cell during the infection process. Most of these studies dealt with *Escherichia coli* phages. Therefore, the following section focuses mainly on these phage systems. The section is organized according to the models for the translocation of the nucleic acid during the infection process, and these models were derived from the analysis of infections by small, medium-sized, and large phages. Figure 1 presents sketches of the different models which will be discussed.

Zones of Adhesion as Sites of Phage DNA Passage

Phages adsorb to specific receptors on the bacterial cell surface (41, 180, 254). After irreversible binding of a phage to the receptor, the phage nucleic acid has to be translocated across the outer membrane, the periplasmic space, and the inner membrane of gram-negative bacteria to reach the cytoplasm. Early electron-microscopic observations led to the suggestion that some phages, such as T1 and T4 (and possibly other phages of *E. coli*), infect the cells at the zones of adhesion, also called Bayer bridges (21). These zones result from a local adhesion between outer and inner membranes and are visible particularly after plasmolysis of cells in 10 to 20% sucrose. Additionally, immunoelectron microscopy during studies on the localization of some membrane-associated proteins (e.g., thioredoxin and penicillin-binding protein) demonstrated that gold particles were clustered at several sites bridging the periplasm and thus seem to connect the inner and outer membranes (25, 26). The number of these adhesion sites was estimated to be about 200 to 400 per cell (22). It was thought that the adhesion sites play a principal role in the transport of membrane proteins and precursor molecules from the cytoplasm into the outer membrane. The advantage of these adhesion sites would be that the transported molecules would remain in a hydrophobic environment without the need for protection from degradative periplasmic enzymes.

Other authors have challenged the idea of adhesion sites and have argued that they result from the method of fixation for electron microscopy (126, 153). If chemical fixation is replaced by cryofixation, no adhesion sites are observed. Kellenberger (153) proposed that the clustered gold label during immunoelectron microscopy derived from a continuous flow of the protein molecules from discrete secretion sites in the inner membrane. Since the periplasm has a gel-like consistency, the proteins do not diffuse through it but remain confined to a stream moving toward the outer membrane. It is not excluded that the stream is flowing along some tiny bridges connecting the membranes. The nature of these hypothetical bridges is not known, but it is speculated that they contain lipids. Nevertheless, the term "adhesion sites" has persisted in the literature, although the term "bridges" would be more adequate to describe the connections between inner and outer membrane observed under particular conditions. Therefore, the term "adhesion site" is also used in this review.

If there are no preexisting adhesion sites, they may be formed during phage infection. This has been suggested for phages T4 and T5. Furukawa et al. (101, 102) proposed that after adsorption and sheath contraction of phage T4, the tail needle is inserted into the outer membrane. In the presence of a membrane potential, the outer and inner membranes may merge so that the tip of the tail central core can come into contact with the inner membrane and suck it to the outer membrane to form an adhesion site (Fig. 1A). Recently, Tarahovsky et al. (289) have reinvestigated the position of the outer and inner membrane at the sites of phage T4 infection and presented a modification of this model (Fig. 2). During contraction of the tail, the central core is proposed to cause invagination of the outer membrane (Fig. 2B and C), which then triggers localized fusion with the inner membrane and forms a hole (Fig. 2D). In agreement with this, Bayer has never found fusions of the membranes in uninfected cells (24). During this stage of infection, a transient ion leakage is observed (see below). Subsequently, a hydrophobic interaction of phage core proteins with membrane lipids could seal the space between the fused membranes and the phage tail core (Fig. 2E). This could be required to allow the passage of the hydrophilic DNA through the hydrophobic environment. An adhesion of the membranes mediated by the phage tail seems possible only in energized cells. In cells treated with a depolarizing poison, e.g., KCN, the phage DNA was injected into the periplasm (149). It was concluded that the depolarization increased the distance between the inner and outer membranes, which no longer allowed induction of membrane fusions by the adsorbed phage. The penetration of phage nucleic acids through zones of localized fusions would not require any specific pores or channel proteins, since in this case the DNA does not really cross the membranes or the periplasmic space because it passes through the central core of the phage tail (Fig. 1A).

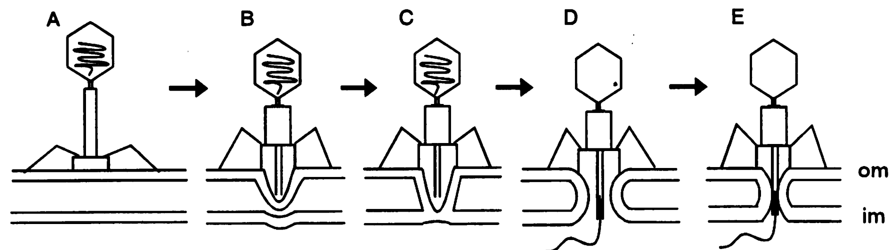

FIG. 2. Induction of membrane fusions by bacteriophage T4 infection. (A) Adsorption. (B) Invagination of the outer membrane (om) to reach the inner membrane (im). (C) Membrane fusion. (D) Injection of DNA, phase of K^+ efflux as a result of leakiness. (E) Sealing reaction between membranes and phage tail. Modified from reference 289 with permission of the publisher.

Another example of induced adhesion sites may be the infection by phage T5. After fractionation of membranes from T5-infected cells in a sucrose gradient, a band of intermediate density was found between the bands of the outer and inner membrane fractions (114). This fraction was thought to contain the contact sites between inner and outer membranes induced by the phage. Accordingly, if phage infection was performed at 4°C, a temperature at which DNA is not injected (159), such an intermediate fraction was not observed (114). Another explanation for the presence of an intermediate fraction could be that it contains inner and outer membranes which are connected by the inserted tail (Fig. 1B; also see below).

Transmembrane Channels for the Translocation of Phage DNA

The hypothesis of a channel-mediated transport of phage DNA has been put forward repeatedly, mainly on the basis of the observation of permeability changes of the cytoplasmic membrane after infection with various phages (T1 [154], T4, [150], T7 [225], LL-H [5], P22 [290], T5 [36]). The permeability changes are explained in different ways. They can be a result of a leakage at the induced membrane fusion sites, for example because the sealing between the phage tail and the membrane is incomplete (289) (Fig. 2D). Ponta et al. (225) propose that the decrease in intracellular ionic strength resulting from leakage provides the optimal conditions for the function of phage enzymes. Keweloh and Bakker (154) assume that the ion efflux leads to depolarization of the cell, which in turn allows entry of the DNA polyanion without the need for overcoming the membrane potential. On the other hand, the following observations provide evidence that the K^+ efflux is due to the opening of a pore which is involved in DNA translocation. After infection with intact T4 particles as well as with ghosts (phage particles devoid of DNA), an efflux of K^+ ions, measured with a potassium-selective electrode, was observed (35). Since the rate of K^+ efflux increased linearly with the multiplicity of infection (from 0.5 to 5), it was concluded that each phage induced one pore. More convincing, the channels induced by intact phage particles seemed to be only transiently open (for about 2 min), whereas those induced by ghosts remained open until cells were depleted of potassium after about 4 min. During the efflux of K^+ there was a simultaneous influx of H^+, Na^+, and Li^+, which eventually led to a breakdown of the membrane potential. When DNA translocation during infection with intact phage particles was completed, the channels were apparently closed, and the cells were repolarized. Perhaps the channel closure was performed by a so-called pilot protein. The pilot proteins are thought to be attached to the DNA end which enters the cell first and to be involved in the high specificity and efficiency of DNA translocation across the cell envelope (155). The pilot protein of phage T4 may be gp2 (181). Gene 2 mutants adsorb and inject their DNA, but the DNA is subsequently degraded. The gene 2 mutation is suppressed in *recBCD* mutant hosts (265). This suggests that gp2 binds to the ends of double-stranded T4 DNA and prevents degradation by the RecBCD enzyme. The basic character of gp2 (18% basic amino acids) supports the idea of DNA binding, but it has not yet been shown by experiment (181). Therefore, it cannot be decided whether gp2 is attached to one end (e.g., that translocated at first) or to both ends. A function during closing of the channel for DNA translocation could be more easily imagined if the protein is also bound to the end which is translocated last.

As channels are induced by both intact T4 phages and ghosts, their formation or opening probably does not depend on the presence of DNA and the pilot protein. In the experiments of Boulanger and Letellier (35), the extent of K^+ efflux correlated not only with the number of phages but also with the extent of DNA injection. After infection of a cell with phage T4, a phage-encoded protein is produced which inhibits the complete injection of the DNA of a second infecting T4 phage (superinfection exclusion [13]). If protein synthesis is prevented by the addition of chloramphenicol during the first infection, the superinfecting phage can inject its DNA into the cytoplasm. A comparison of the K^+ efflux and the DNA penetration during superinfection experiments (without chloramphenicol) showed that during the second infection, when DNA is presumably not translocated into the cytoplasm but is degraded in the periplasm, the ion efflux was strongly decreased (by a factor of about 4). When chloramphenicol was present during the first infection, the K^+ efflux during the second infection was as high as during the first infection. Moreover, the rates of K^+ efflux and of DNA translocation showed the same temperature and voltage dependence (36). Infection was successful only at temperatures above 14°C and required a membrane potential of at least 85 mV (35, 161). It is not clear whether the insertion or the opening of the putative channel is inhibited at low temperatures. These observations suggest that the DNA translocation pore which is functional during T4 infection changes its properties during the infection process. It may be closed before the DNA passes through it, it triggers cytoplasmic ion release concomitant with DNA translocation, and at the end it is closed again. So far, direct proof for a pore-forming protein involved in T4 DNA injection has not been obtained, and the above observations are also in accord with the model of DNA translocation through holes derived from induced membrane fusions. It is still not known whether the putative T4-induced channel is formed by phage and/or host proteins and whether the channel for ion efflux and for DNA translocation are identical. It is also possible that the

DNA translocation induces or opens ion channels in the neighborhood.

Potassium efflux and the temperature dependence of the insertion of the channel for DNA translocation were also observed during T5 infection (36). T5 injects its DNA into the cell in a two-step process (171). Potassium efflux also occurred in two steps (36). In the first step of DNA injection, 7.9% of the DNA, which is called first-step-transfer DNA, enters the cell. Two genes (A1 and A2) located on the first-step transfer DNA must be expressed before the remaining DNA can be translocated into the cytoplasm. During synthesis of the two pre-early proteins A1 and A2, DNA translocation is arrested and the channel remains closed. When the synthesis of A1 and A2 was prevented by chloramphenicol, the second K^+ efflux did not take place. It is likely that proteins A1 and A2, which are found in the envelope and which bind to DNA (84), are involved in the reopening of the channel.

Phage-specified pore-forming proteins. Phage T5 and fd-encoded pore-forming proteins have been identified. These proteins were isolated and tested for their ability to form pores in artificial lipid bilayer membranes.

The pore-forming protein of phage T5, the product of gene 2 (pb2), has been purified from phage tails (95). This protein, with an apparent molecular mass of 108,000 Da, forms the central tail fiber of T5. The receptor-binding protein (pb5) necessary for phage adsorption is not part of the tail tip but is located at the distal part of the tail fiber near the attachment site between the straight tail fiber and the conical part of the tail (122). Therefore, it is assumed that the tail fiber dives into the outer membrane to allow binding to the receptor protein provided by the *fhuA* gene (formerly known as *tonA*) of the host cell. The tail protein pb2 then forms water-filled transmembrane channels (95). In black lipid bilayer membranes it forms pores with an estimated diameter of about 2 nm, which would be large enough for the passage of a double-stranded DNA helix. A channel formed by five or six molecules of pb2 could span the outer membrane, the periplasmic space, and the inner membrane (Fig. 1B). The channel would be long enough to connect the outer and inner membranes even in unenergized cells. This may explain why T5, in contrast to T4, injects the first-step-transfer DNA even into KCN-treated cells (185). However, closing of the channel and reopening for the injection of the second-step DNA are prevented in depolarized cells (36).

A pore-forming protein has also been isolated from the filamentous phage fd (105). It is encoded by gene 3 (gp3). Three knobs at one end of the phage filament are formed by gp3 and function as the adsorption site for binding to the F pilus (110). gp3 of phage fd has been purified and shown to produce transmembrane channels in artificial lipid bilayer membranes (105). This is in accord with the finding that the protein (406 amino acids) contains several putative membrane-spanning regions (27). The protein is able to oligomerize and to form a pore with an estimated diameter of 1.6 nm, which would allow the translocation of the circular single-stranded fd DNA helix with a diameter of about 0.85 nm. In addition to its presence on phage particles gp3 has also been localized in the outer membrane of infected cells. On the basis of these findings, the infection by fd is proposed to proceed as follows. Phage fd adsorbs to the F pilus tip with help of receptor protein gp3. After pilus retraction, the phage reaches the cell surface. Contact of gp3 with the outer membrane initiates a conformational change in the protein, resulting in its insertion into the membrane and in oligomerization. Thus, gp3 forms a pore only in the outer membrane. The translocation of fd DNA through the inner membrane is probably mediated by the *tolQRA* products (see below) of the host cell (Fig. 1C).

The pore-forming proteins of phages T5 and fd are constituents of the phage capsid. Especially for the tailed phages, it can be easily imagined that the hollow tail itself inserts into the membranes to form a pore for translocation of DNA into the cytoplasm (Fig. 1B). An alternative mechanism is assumed for phage P22. Phage P22 has a short tail, not long enough to cross the cell envelope (118). However, in the capsid of P22 three minor proteins are tightly associated with the DNA (34, 45), and one of these (gp16) plays an essential role in the translocation of DNA into the cell during infection. Although gene 16 mutant phage particles are unable to inject their DNA into the cell, it is possible to complement gp16-defective particles at the membrane by preinfection of host cells with a helper phage (127, 128). Therefore, it was suggested that the protein gp16 forms or induces a pore or modifies an existing pore for DNA translocation (106, 160). This pore, once formed by one phage, may be used during a limited time span (about 10 min after the first infection) by other phages. Recently, gp16 has been purified and characterized with regard to its participation in procapsid assembly (291). Studies on pore formation have not yet been performed. The nucleotide sequence of gene 16 argues against the hypothesis that gp16 itself may form a membrane pore, since the deduced amino acid sequence does not contain potential transmembrane regions (303). The observation that gp16 always copurifies with gp20 suggests that the latter protein may also be involved in pore formation (291).

Participation of host proteins in phage DNA translocation. As mentioned above, certain host proteins participate in the injection process of phage fd DNA. The *tolQ*, *tolR*, and *tolA* mutants of *E. coli* cannot be infected by phage fd and other phages which use the tips of conjugative pili as receptors (39, 246). These mutants have a pleiotropic phenotype (for a review, see reference 319). They are hypersensitive to certain antibiotics and detergents and are tolerant to group A colicins (the colicins are divided into two groups: colicins A are inactive against *tolA* mutants, and colicins B are inactive against *tonB* mutants). The precise functions of the Tol proteins are not known, but it seems that they stabilize the outer membrane and are perhaps involved in membrane assembly (178). TolA is well characterized. It is a membrane protein of 421 amino acids with a molecular mass of 44.2 kDa (176, 177). It apparently has three domains. The N-terminal domain, of 34 amino acids, anchors the protein in the inner membrane. The second domain following the membrane anchor forms a long alpha-helix of 230 amino acids. The third domain, of 120 amino acids at the carboxyl terminus, seems to be responsible for TolA function, e.g. uptake of colicins A. It is proposed that the helical part of the protein spans the periplasm so that the C-terminal part of the protein can reach the outer membrane and interact with the receptor-ligand complex (receptor/colicin or receptor/phage). Less is known about the proteins TolQ and TolR. TolQ (formerly Fii) is a membrane protein of 230 amino acids (25.5 kDa) with three membrane-spanning regions (287). It shows a rapid turnover in the membrane and is thought to leave stable species only at the adhesion sites between the membranes (37). TolR is a protein of 141 amino acids (15.5 kDa) and has one potential membrane-spanning region, but it is not yet known whether it is located in the membrane (287). It is not known whether one or several of the Tol proteins can form pores for the translocation of colicins or phage DNA. It may well be that the Tol proteins only guide colicins or phage DNA from the receptor site to the actual site of translocation through the inner membrane. During this process, gp3 of phage fd seems to interact directly with one or several Tol

proteins. This was concluded from the observation that cells producing gp3 from a plasmid can no longer be infected by fd and show an increased tolerance to colicins (33). Apparently, the plasmid-borne gp3 can leave the cytoplasm and associate with the Tol complex, which is then no longer free to interact with phage-derived gp3 or colicin.

Whereas the phages discussed so far probably do not need a channel provided by the host cell for the translocation of DNA across the outer membrane, phage lambda is an example of the translocation of DNA across both the outer and inner membranes with the help of pores produced by bacterial proteins. Phage lambda binds to LamB, located in the outer membrane, which is a component of the maltose transport system (288). The protein has a molecular mass of 47 kDa, and three subunits form a channel for the selective transport of maltose and maltodextrins (56, 184, 219). This pore formation by LamB led to the speculation that LamB may also be the pore for lambda DNA translocation across the outer membrane. There was some experimental evidence in favour of this idea. When liposomes carry inserted LamB protein, bacteriophage lambda injects its DNA (240). The necessary transmembrane channels were formed or opened only by intact phage particles. Neither purified tails nor ghosts were able to induce such channels (212, 240), suggesting that phage tail proteins are not involved in pore formation or that the DNA or the pilot protein is necessary for channel formation and/or opening by phage tail proteins, respectively. Perhaps the entrance of DNA into the phage tail, after unplugging of the distal part of the tail, triggers channel formation or channel opening by tail proteins. The tail fiber itself, consisting of phage protein gpJ, is responsible only for binding to the LamB receptor. It seems not to dive into the membrane, because it remains sensitive to pronase and proteinase K after adsorption (241).

Although LamB is clearly involved in lambda infection, there is no evidence that it is the pore for DNA translocation itself. The LamB pore was estimated to have an inner diameter of only about 0.7 nm, which is not sufficient for translocation of double-stranded DNA (29). Therefore, it is doubtful that lambda DNA is translocated across the outer membrane through a lamB pore. On the other hand, LamB suffices for the injection of DNA into liposomes; further host proteins are not required for this DNA translocation. Presently, two hypotheses for the necessity of only LamB and the phage tail for DNA translocation across the outer membrane are conceivable: (i) the hollow tail (inner diameter, 3 nm) composed of protein subunits encoded by gene V (152) inserts into the outer membrane in the neighborhood of LamB (Fig. 1C); and (ii) interaction of LamB with phage tail proteins leads to an extension of the LamB pore (Fig. 1D). Experimental evidence for these possibilities is not available.

If lambda DNA is in fact injected through a pore in the outer membrane, it has to cross the periplasm (Fig. 1C and D), where it would be a target for endonuclease I. Early studies (89) have shown that phage lambda infects wild-type *E. coli* cells as effectively as it infects mutants defective for endonuclease I. Therefore, if translocation of DNA across the periplasm occurs, an efficient yet unknown mechanism of protection against endonuclease I must exist.

Studies on the translocation of lambda DNA across the inner membrane suggest that tail proteins are involved (see below), besides bacterial proteins. In early experiments it was found that translocation of lambda DNA across the inner membrane was blocked in mutants with a defective *pts* operon (phosphotransferase system). Mutants (termed *pel* for penetration of lambda) that abolish lambda propagation by the inhibition of injection of the DNA were isolated (249, 250). During mapping of the *pel* mutations it became apparent that they lie within the *ptsM* gene (91). The *pts* operon consists of three genes encoding the three subunits of mannose permease (93). Subunit III^{Man} is a hydrophilic protein of 35 kDa, subunit $II\text{-}P^{Man}$ is a very hydrophobic protein of 28 kDa, and subunit $II\text{-}M^{Man}$ is a moderately hydrophobic protein of 31 kDa. Proteins II-M and II-P constitute integral membrane proteins and are usually copurified. All three subunits are required for mannose transport; however, subunits II-P and II-M together are sufficient for lambda DNA translocation. Partial deletion of the genes of these two subunits, which resulted in truncated proteins, reduced the infectivity of phage lambda. The effect is more significant (infectivity decreased by 2 orders of magnitude) with truncated II-M than with truncated II-P (infectivity decreased by 1 order of magnitude). Possibly II-M protrudes into the periplasm, which would facilitate an interaction with the phage DNA and/or phage tail proteins. It was proposed that II-M and II-P form the pore in the cytoplasmic membrane for transport of sugar and lambda DNA (93). No data are as yet available on the inner diameter of the pore and on the factors that cause the pore to open.

Phage-encoded proteins also appear to be involved in DNA translocation through the inner membrane. Mutants of phage lambda, termed *hp* mutants, which are able to infect *pts* mutants of *E. coli* have been isolated (250). The *hp* mutations were mapped within genes V and H. Protein V is the major tail protein which forms the tail tube consisting of 32 hexameric rings (47, 48, 152). The gpH, specifically the proteolytically processed form gpH*, is the pilot protein of phage lambda engaged in the DNA injection process (123). gpH* may also be involved in pore formation, because its protection from protease degradation after complexation with liposomes carrying the *Shigella* receptor protein (LamB) indicates that gpH* is inserted into the lipid bilayer (241). The amino acid sequence of gpH* indicates two possible transmembrane regions. Furthermore, sequence homologies between gpH* and gp2 of phage T5 (see above) support the idea that gpH* is involved in the translocation of phage lambda DNA into the cell (121).

In this section, several models of the translocation of DNA across the bacterial membranes during phage infection have been presented. There is the model of a phage-induced fusion of the inner and outer membranes, which would allow DNA injection without crossing of the membranes or the periplasmic space (T4 [Fig. 1A]). Alternatively, there are several different models for DNA translocation across the membranes via protein channels. The channel provided by the phage tail can be long enough to span the whole envelope (e.g., T5 [Fig. 1B]), or else there are different channels in the inner and outer membranes (e.g., fd, lambda) which may derive from the phage and/or the host cell (Fig. 1C and D, respectively). In these cases, DNA translocation across the periplasmic space would be necessary. It is possible that the different morphologies and mechanisms of the phage tails, especially the length of the tail, its ability to contract, the structure of the core, and the potential to make contact with the cytoplasmic membrane, result in different DNA translocation mechanisms. It is not excluded that further studies will lead to a more homogeneous general model, which may be a synthesis of those described here (Fig. 1).

DNA TRANSLOCATION DURING BACTERIAL CONJUGATION

Bacterial conjugation is a process by which DNA is transferred from a donor to a recipient across the envelope of both cells. Without exception, it is mediated by plasmids and is an

effective mechanism for the dissemination of these conjugative plasmids. The phenomenon of conjugation was first observed in 1946 in cultures of *E. coli* (174). Although conjugation systems have been studied in detail only in gram-negative bacteria, they have also been identified and analyzed in gram-positive bacteria, e.g. *Streptomyces* spp. (131), *Streptococcus* spp. (57, 60), and *Bacillus* spp. (20), and in archaebacteria (242). Conjugation occurs not only between closely related bacterial species but also between different genera and even between gram-negative and gram-positive organisms (44, 192, 302). Conjugative DNA translocation is not restricted to bacteria. It has been shown that conjugative plasmids of *E. coli* can even mobilize DNA to the yeast *Saccharomyces cerevisiae* (120). Thus, conjugation has the broadest host range among the mechanisms for interbacterial genetic exchange (conjugation, phage infection and transduction, transformation). The best-studied model of a conjugative plasmid is the F factor of *E. coli*. Therefore, the following section focuses mainly on what is known about the translocation mechanism of the F factor. The conjugation system of F-like plasmids was subject of several reviews (137, 323–325). A comprehensive collection of reviews dealing with bacterial conjugation has been published recently (58).

Processing of F-DNA during Bacterial Conjugation

This section will summarize the events and components of DNA processing which are relevant for the translocation of F-DNA. The genes involved in conjugation are organized in a single operon, the *tra* operon (138). The *tra* operon consists of 35 open reading frames (ORFs) (*tra* and *trb*).

Early studies have shown that the F factor is transferred from the donor to the recipient cell as a linear single strand with the 5′ terminus ahead (61, 136, 214). Although it was formerly thought that nicking of the F factor at *oriT* was catalyzed by the products of the genes *traI* and *traZ* (94), there is more-recent evidence that TraZ is the product of an in-frame translational restart within the *traI* gene (300). TraI binds to *oriT*, nicks the DNA strand which is transferred, and remains covalently bound to the 5′ terminus of the nicked strand (190, 191, 237, 301). Besides the endonuclease activity, TraI has helicase activity and unwinds F-DNA during conjugal translocation (1). The energy for strand separation is provided by ATP hydrolysis (157). The purified TraI protein exhibits two ATPase activities (66), and there are two ATP-binding sites in the protein as indicated by the nucleotide sequence (40). The specific roles of the two putative ATPases are not yet known. The covalent binding of TraI to the 5′ end of the nicked strand and a requirement for protection of the linear DNA single strand against exonucleolytic attack led to the suggestion that TraI may be transferred together with the DNA into the recipient cell. It has also been suggested that TraI may have a ligation activity by which it might reseal the nicked strand in the recipient cell (94, 191). An alternative model proposes that TraI is sequestered in the donor cell and that the DNA enters the recipient cell in a single-stranded but end-less form (191). The "end-less DNA" model reconciles all requirements for conjugative DNA translocation: (i) an end-less DNA does not need protection against exonucleolytic attack; (ii) TraI is a cytoplasmic protein which is associated with the membrane when overproduced together with TraD, indicating a functional interaction between TraI and TraD in the donor cell (66); (iii) association of TraI with the cytoplasmic membrane would result in a net movement of the single-stranded DNA relative to the cell surface catalyzed by ATP hydrolysis (264); and (iv) TraI could not be detected in the recipient cell by immunological methods, contradicting the hypothesis that TraI enters the recipient cell (232).

Two further proteins are involved in DNA processing. TraY binds to the *oriT* region and is thought to be a component of the endonuclease, although neither a nicking activity nor an interaction with TraI has been detected (170). The product of *traM* of plasmid R100 and the F factor also binds to DNA at the *oriT* region (2, 72, 253). Because the protein was also found in association with the membrane, it is assumed that TraM functions as a membrane anchor for *oriT* which is transferred last during conjugation. The *oriT* is anchored in the membrane, probably near the pore for DNA translocation (2, 3). Another role of TraM may be to initiate the DNA translocation after receiving a hypothetical signal produced upon successful mating-pair formation (324).

The question arises whether any of these Tra proteins which bind to DNA escort the DNA during translocation through the membranes, e.g., to protect it against nucleases or to serve as a pilot protein. Such a DNA-associated protein transport was proposed for the single-stranded-DNA-binding protein (SSB protein) encoded by the F factor, which may cover the transferred DNA (324). Coating of single-stranded DNA by SSB protein was also proposed to occur during T-DNA translocation (see below). It has been demonstrated that during the translocation of the conjugative plasmid CoIIb-P9, large amounts of the Sog protein, which has a primase activity, are transported from the donor to the recipient cell, suggesting that the transferred single-stranded DNA is coated by the Sog protein (231). However, translocation of Tra proteins from the donor to the recipient cell during F-mediated conjugation was not found (232). The only protein from the donor cell which was detected in the membrane fraction of recipient cells after conjugation was a 92-kDa protein. As mentioned above, immunological tests showed that it is not a processed form of TraI with a molecular mass in this range. Therefore, the origin of this protein remains unclear. It should be tested whether this protein is TraD. TraD has a molecular mass of 82 kDa, is a membrane-associated protein, and possibly functions as a pore or as part of a pore for DNA translocation in the donor cell (220, 221) (see below). Thus, it could be that TraD also takes part in the pore of the recipient cell.

F Pilus—an Organelle for DNA Translocation?

Since the discovery that *E. coli* cells carrying the F factor synthesize F pili (14), it has been a matter of debate whether the DNA is transferred through the pilus. Pili are determined by conjugative plasmids of all incompatibility groups in *E. coli*; they belong to three different morphological groups, which are described as thin flexible, thick flexible, and rigid filaments (38). The F pilus is a hollow cylinder of about 20 μm in length, 8 nm in outer diameter, and 2 nm in inner diameter, which is large enough for the passage of single-stranded DNA (98). The arrangement of the pilin protein subunits in the pilus is similar to that in the coat of filamentous phages (98). A structural similarity to the tail of T phages also exists, leading to the "sex-virus infection" model of conjugation (reviewed in reference 43). The hollow-cylinder model of DNA translocation does not discriminate between the translocation of a DNA single strand (conjugation), a circular single strand (fd infection), and a double strand (T phage infection). If the pilus is anchored in the inner membrane at the zones of adhesion as suggested by Bayer (23), it would have all the characteristics of an organelle for the DNA translocation. A recent publication presents evidence for direct DNA translocation through extended F pili (117). Donor and recipient cells in a conjugation

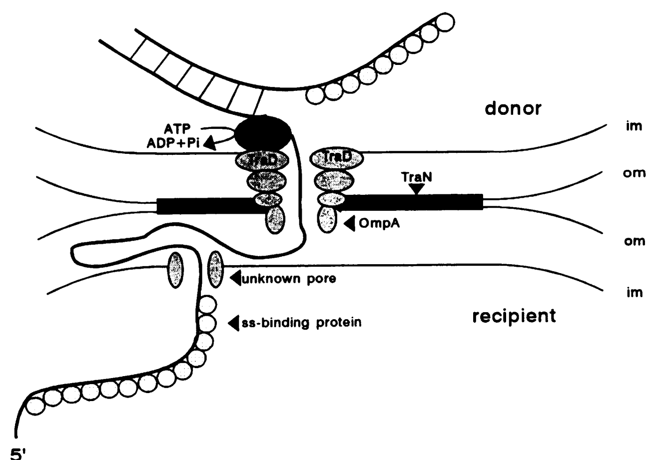

FIG. 3. Transfer of single-stranded (ss) DNA of the F factor across the membranes of donor and recipient cells during conjugation. The transmembrane channel of the donor cell may be composed of different Tra proteins. Modified from reference 88 with permission of the publisher.

experiment were separated by a Nuclepore filter. The filters were thick enough to prevent direct cell-to-cell contact and had pores from 0.01 to 0.1 μm in diameter to allow the passage of extended F pili. In these experiments, conjugative translocation of F-DNA was found. However, the translocation rate was very low, suggesting that DNA translocation through an extended pilus may rather be an exception. Other observations led to the opposite suggestion, namely that the pilus is needed only for the initial contact between the mating partners and that DNA translocation occurs during close cell-to-cell contact. Marvin and Hohn (189) and Jacobson (139) reported the retraction of pili after adsorption of filamentous phages. Sorting of close and loose mating pairs resulted in a higher frequency of transconjugants with close mating pairs (217). Disaggregation of pili by addition of sodium dodecyl sulfate (SDS) does prevent successful conjugation when the SDS is added before the formation of close mating pairs but has no effect when the SDS is added at the stage of close cell-to-cell contact (4). Perhaps the pili lead to the right positioning of the mating partners and, upon retraction, bring together the sites of the cell surfaces where the DNA translocation from the donor and recipient cells takes place. The actual DNA translocation sites should then be located in the neighborhood or even at the basis of the pilus.

F-DNA Translocation during Stable Cell-to-Cell Contact

Proteins of the donor cell. A model for F-DNA translocation during stable cell-to-cell contact was proposed by Dürrenberger et al. (88). After examination of conjugation-specific contacts by electron microscopy of ultrathin sections of various stages of mating-pair formation, they identified an electron-dense dark layer between the outer membranes in the area where the conjugating partners attach; they termed this layer the conjugational junction. No such structures were seen at regions of close contact between only donors or only recipients. Conjugational junctions may contain the TraN protein, because TraN is known to be an outer membrane protein essential for mating-pair stabilization (186). No further substructures in the bacterial envelope, like channels or fusions between the membranes, could be detected. A model of conjugative DNA translocation is summarized in Fig. 3 and accommodates a variety of other observations. Export of the F-DNA from the donor cell proceeds through a pore formed by or with participation of the TraD protein. The 82-kDa TraD protein was formerly assumed to be located in the inner and outer membrane of F^+ *E. coli* cells (3, 199) but then was shown to be located only in the inner membrane (140, 221). Temperature-sensitive *traD* mutants form stable mating pairs at the nonpermissive temperature but do not translocate DNA. When the temperature was shifted from 42 to 32°C, TraD function was restored, as shown by the observation that DNA translocation occurred (220). Thus, TraD seems to play a direct role in F-DNA translocation. In this context it should be mentioned that the *traD* nucleotide sequence contains a consensus sequence for an ATP-binding site and that the purified protein has a DNA-dependent ATPase activity. Several regions of unusually high pI (pI > 10) (140) and binding of the purified protein to DNA-cellulose (221) indicate the ability of TraD to interact with DNA. It is also assumed that the TraD protein may serve as a membrane anchor for the *traI* gene product, because in the presence of TraD the cytoplasmic TraI protein was found to be membrane associated (see above) (66). In summary, genetic data and characteristics of the protein suggest that TraD may function as a nonspecific pore for the export of DNA from the donor cells (300), but there is no experimental evidence that it is a pore-forming protein. The recent findings that TraD is located only in the inner membrane contradict the hypothesis that TraD may span both membranes. Characterization of further Tra proteins will show whether perhaps other proteins take part in a more complex transmembrane channel structure. One candidate may be the TraC protein, which is involved in pilus assembly (251). Because *traC* mutants showed a severely reduced conjugation efficiency while mating-pair formation was not greatly affected, it was concluded that TraC may also play a direct role in DNA translocation, e.g., as part of a membrane-spanning Tra protein complex. Recent experiments show that TraC is membrane associated only in conjunction with other Tra proteins (252).

Several of the numerous *tra* gene products have been shown to be membrane proteins or membrane-associated proteins. Most of them (at least 12 [322]) are involved in pilus assembly. They have not yet been characterized to an extent which allows us to conclude whether they are part of a DNA transport apparatus.

Proteins of the recipient cell. Little is known about the conjugal DNA translocation across the membranes of the recipient cell. Two classes of *E. coli* mutants defective in envelope functions are known also to be defective as recipients in conjugation. These mutants, formerly termed *con* mutants, either lack the major outer membrane protein OmpA or have an altered lipopolysaccharide (188, 267). The defect in the lipopolysaccharide may have a direct effect or may abolish the correct insertion of OmpA into the outer membrane. This or a mutation in the *ompA* gene prevents the formation of stable mating pairs. A complex of purified OmpA and lipopolysaccharide inhibits F-pilus-mediated conjugation (305). Indirect evidence for the participation of OmpA in mating-pair formation or stabilization is provided by studies on the mechanism of surface exclusion (239). Surface exclusion is the reduced ability of a F^+ cell to function as the recipient in conjugation and depends on the gene products of *traS* and *traT* (3, 4). TraT is an outer membrane lipoprotein. Because TraT shares some amino acid sequence homology with the tail protein gp38 of some OmpA-specific phages (e.g., T2 and K3) and inhibits the adsorption of these phages when added to cells which produce TraT from a plasmid, it seems that TraT can interact with

OmpA (239). It is assumed that TraT binds to surface-exposed domains of OmpA and thus abolishes its receptor function required for mating-pair formation and phage adsorption. However, it has not yet been shown experimentally that the pilus tip of the donor cell contacts with the OmpA protein or some other surface components of the recipient cell. If OmpA really is the receptor for the pilus, the ligand of the donor cell seems to recognize only a very small part of the OmpA protein since *ompA* point mutants deficient in conjugation all have one unique amino acid substitution (238).

It was previously considered that OmpA may form a pore or not (29, 187, 210). Recently, it was demonstrated that OmpA forms a nonspecific diffusion channel. The pore size was estimated by the liposome-swelling technique to have a diameter of about 1 nm (286) and was shown by conductance measurements to be in the range of 0.6 to 0.7 nm (248). This could be wide enough for the passage of single-stranded DNA. Accordingly, OmpA could have two functions: it may serve as receptor for the F pilus and thus would be involved in the positioning of the mating partners, and it may also provide the pore for the DNA translocation through the outer membrane of the recipient cell.

There are no experimental observations or models which help us to understand the translocation of DNA across the inner membrane of the recipient cell. One approach could be the isolation and characterization of further conjugation-defective recipient mutants. In the past, the screening for such mutants has revealed only *ompA* mutants. It may well be that mutants defective for the hypothetical pore protein in the inner membrane are inviable because this protein has another essential function(s) besides DNA translocation. Perhaps a search for conditional mutants would be successful.

tra Regions of Broad-Host-Range Plasmid RP4

The F plasmid has a narrow host range. Of the broad-host-range conjugative plasmids, RP4 has been most extensively studied. The *tra* genes of RP4 are completely sequenced (175, 336). Although the conjugative processes are very similar to those of the F factor and although the proteins involved in mating-pair formation, in DNA processing, and in translocation have analogous functions, no significant amino acid sequence homologies between F and RP4 Tra proteins have been found (Table 1). The *tra* genes of the F factor are clustered in a single operon, whereas those of RP4 are organized in two separate regions (TRA1 and TRA2). The TRA1 region contains *oriT* and mainly the genes for the DNA translocation metabolism (*tra* genes [336]). DNA translocation is triggered by the introduction of a nick at *oriT* following the assembly of a specialized nucleoprotein complex (the relaxosome) of TraI, TraJ, and TraH with *oriT*. After nicking, TraI remains covalently bound at the 5′ end of the nicked strand and may facilitate the translocation of DNA from the donor to the recipient (100, 222, 223). At least two genes of the TRA1 region, *traG* and *traF*, encode proteins which are probably components of the DNA translocation apparatus. This was concluded from the amino acid sequences, which suggest that TraG and TraF are membrane proteins (318). Most of the proteins which may take part in mating-pair formation and the DNA translocation apparatus are encoded within the TRA2 region (*trb* genes [175]). In the core region of TRA2, which is essential for conjugation in *E. coli*, 12 ORFs were found, and 11 corresponding proteins were identified in *E. coli* (175). Most of these proteins are hydrophobic, supporting the idea that they are involved in mating.

Since the nucleotide sequences of the *tra* regions of the F factor and of RP4 are completely known, characterization of the proteins is in progress, and additional data concerning the DNA translocation machinery of the donor may be expected soon. A new experimental field will be devoted to the requirements for a cell to become a recipient partner in conjugation. Because conjugation is not limited to a variety of gram-negative strains but also occurs between gram-negative and gram-positive bacteria and between bacteria and eukaryotic cells (for reviews, see references 12, 119, and 192), it will be interesting to know whether there is a common receptor and perhaps even a common translocation apparatus for the translocation of donor DNA.

CONJUGATIVE TRANSPOSITION

Another process of DNA translocation from cell to cell is conjugative transposition (for reviews, see references 59 and 255–257). Conjugative transposons are found predominantly in gram-positive bacteria but also occur in gram-negative bacteria. These transposons are transferred from one bacterial cell to another, probably in the form of a double-stranded covalently closed circular intermediate which can be isolated from the cells (258). The mechanism of DNA translocation from one cell to another is completely unknown. The transposons are not large enough (the smallest is Tn*925* and has about 16.4 kb) to encode the numerous Tra functions known for the conjugative plasmids. Conjugative transposition of Tn*925* is unique since an extensive mobilization of chromosomal genes and nonconjugative plasmids occurs. This led to the hypothesis that during transposition the mating pair may resemble a state of protoplast fusion, with a transient diploid stage which allows extensive genetic exchange (299). However, this phenomenon is not observed with other conjugative transposons, such as Tn*916* (97). The available data suggest that the channel for DNA translocation may differ in size for different conjugative transposons: a larger pore may allow extensive exchange of genetic material, whereas a smaller channel may restrict the exchange.

T-DNA TRANSLOCATION FROM *A. TUMEFACIENS* TO PLANT CELLS

In a variety of plants, infection with the gram-negative bacterium *Agrobacterium tumefaciens* is accompanied by the translocation of a specific segment of bacterial DNA into plant cells (the T-DNA), which leads to tumorigenesis, specifically crown gall formation (32, 130, 144, 209). The T-DNA translocation was the first example of genetic exchange between microorganisms and cells of higher organisms. The expression of the T-DNA after integration into plant nuclear DNA results in an overproduction of phytohormones and thus causes pertubation of cell growth and differentiation (for a review, see reference 201). For translocation of T-DNA from the bacterium into the plant cell, the DNA must be translocated across the inner and outer membranes of the bacterium and the cytoplasmic membrane of the plant cell and finally has to enter the nucleus of the plant cell. The T-DNA is a copy of a specific part of the Ti plasmid of *A. tumefaciens* delimited by two 25-bp imperfect direct repeats at the ends (border sequences). T-DNA translocation requires the induction of the *vir* genes on the Ti plasmid by phenolic compounds (e.g., acetosyringone) which are released by wounded plant cells (68, 273, 274, 278, 326). This interesting system has attracted much attention, not least because of its use in the experimental integration of foreign DNA into plant genomes. Numerous publications and reviews address the processing of T-DNA and the mechanism

of T-DNA transfer (54, 129, 156, 278, 326, 332, 333, 335). As in the preceding section, the section on T-DNA transfer will discuss the structure of the transferred DNA, the DNA-binding proteins which may be cotransferred, and proteins which may contribute to the translocation apparatus.

Conformation of Transferred T-DNA

Whether the DNA is double stranded or single stranded, respectively linear or circular, determines the dimensions of the DNA, the susceptibility to nucleases, and the binding of proteins. It seems likely that these characteristics influence the translocation mechanisms of the DNA species. The conformation of the transport form of T-DNA was repeatedly investigated. In the analysis of DNA from bacteria after induction of the *vir* genes by acetosyringone, linear double-stranded DNA (87, 279), circular double-stranded DNA (10, 11, 329), and linear single-stranded DNA (9, 142, 275, 276) were identified. On the basis of these observations, two hypotheses on the mechanism of the T-DNA translocation were put forward (10). According to the first, two nicks are introduced at the border sites in one strand of the Ti plasmid and the single-stranded T-DNA produced by strand displacement is released and transferred. In the second model, the single-stranded intermediate is converted to a double strand by synthesis of the complementary strand in the bacterial cell prior to transfer. The possibility that cleavage of the linear double-stranded T-DNA molecule from the Ti plasmid occurs is unlikely for the following reason. Although the VirD endonuclease, which is responsible for the generation of T-DNA (see below), produces double-strand breaks under certain conditions in vitro (142), T-DNA generated in this fashion would cause loss of the T-DNA region from the Ti plasmid. Circular DNA seems not to be the transport form, since the translocation of the T-DNA was shown to be polar beginning with the right border sequence (260, 311). The experiments involved deletions of the border sequences. Although deletion of the left border sequence had only little effect, deletion of the right one abolished tumorigenesis. After reintroduction of a synthetic right border, the ability for tumor formation was restored. Bakkeren et al. (16) analyzed T-DNA isolated from plant cells. In their experiment a replicon from the cauliflower mosaic virus was provided with T-DNA border sequences and introduced into *A. tumefaciens* containing a Ti helper plasmid (for a review, see reference 112). After "agroinfection" of host plants, "viral" DNA was isolated and the T-DNA border sequences contained in it were sequenced. A conserved right border sequence and rather variable left border sequences but no perfect hybrid border were found, suggesting that the T-DNA is transferred to the plant in a linear form and that one end (the right border) was protected against degradation. The experiments do not allow us to distinguish between translocation of double- or single-stranded DNA. Nevertheless, in recent publications (e.g., references 54 and 293) the authors agree with the model that the T-DNA is transferred into the plant cell in the form of a linear single-stranded DNA molecule complexed with proteins VirE2 and VirD2 (see below).

Vir Proteins Involved in Processing and Translocation of T-DNA

Processing and translocation of the T-DNA are mediated by gene products of the seven *vir* operons (*virA* to *virE*, *virG*, and *virH*) located on the Ti plasmid but not on the T-DNA itself. Expression of the *vir* genes is positively regulated by the products of *virA* and *virG*, which are activated by the phenolic compounds such as acetosyringone released by wounded plants (277). The products of the *virC* operon (VirC1 and VirC2) enhance the T-DNA processing by binding to the so-called overdrive, a specific DNA sequence adjacent to the right border of the T-DNA (297, 298). The *virH* locus (formerly *pinF*) encodes two cytochrome P-450-type proteins, which may be involved in the detoxification of compounds released at the site of wounding (151). A further gene, *virF*, found only in octopine-type Ti plasmids, determines the host range of *A. tumefaciens* (141, 196). The products of the *virD*, *virE*, and *virB* operons will be discussed in more detail below because they are probably directly involved in the translocation of the T-DNA.

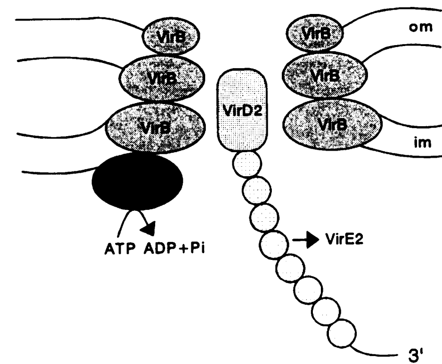

FIG. 4. One-step DNA transfer across the inner membrane (im) and outer membrane (om) of *A. tumefaciens* through a channel composed of different VirB proteins.

Possible functions of the VirD1,2 endonuclease. The *virD* operon consists of five ORFs. Two of them are the genes *virD1* and *virD2*, which encode two proteins with molecular masses of 16.2 and 47.4 kDa, respectively (142, 330). The two proteins are responsible for the endonucleolytic cleavage at the border sites (275, 310, 312). It is possible that VirD2 effects sequence recognition and that VirD1 protein also has a topoisomerase activity (103). In *E. coli* cells expressing *virD1* and *virD2*, VirD2 forms a relaxed nucleoprotein complex with plasmids carrying the T-DNA border sequences, which is comparable to the relaxation complex of conjugative plasmids (96). T-DNA isolated from *vir*-induced bacteria is tightly, probably covalently, associated with VirD2 at the 5′ end. Therefore, it is possible that VirD2 is transferred together with the T-DNA into the plant cell (Fig. 4). There are several hypotheses on the function of VirD2 bound to the 5′ end (87, 124, 132–134, 281, 326, 331): (i) the associated protein may determine the polarity of T-DNA translocation; (ii) the protein may protect the 5′ end of the DNA against exonucleolytic attack; (iii) the protein may serve as primer for complementary-strand synthesis; (iv) the assumed covalent linkage of VirD2 may conserve the energy of the endonucleolytic cleavage for an energy-depending process like religation; (v) if VirD2 does enter the nucleus of the plant cell, it may play a role in the integration of the T-DNA into the plant genome (indeed, there are some sequence similarities between the central parts of the *virD2* gene product and the *E. coli* DNA ligase [326]); or (vi) VirD2 may serve as "pilot protein" to facilitate T-DNA translocation across the bacterial membranes and/or as a nuclear targeting signal guiding the DNA into the plant nucleus. Support for the last of these assumptions is provided by the presence of two nuclear localization signals in VirD2, one at the N-terminal end of the protein and one of a bipartite type at the extreme C-terminal end (134, 244, 310). The nuclear localization signal is a short

consensus sequence of basic amino acids found in several karyophilic proteins (49). Deletion of the C-terminal nuclear localization signal sequences results in a nontumorigenic phenotype of the mutants (281). Fusion of the C-terminal nuclear localization signal sequence of VirD2 with the C terminus of the reporter protein β-glucuronidase results in nuclear localization of the fusion protein in tobacco protoplasts (134). Similarly, a fusion protein of the N-terminal part of VirD2 with the N terminus of β-galactosidase is found in the nucleus of transgenic tobacco plants (124). Therefore, it is feasible that the VirD2 protein also directs the T-DNA to the plant nucleus.

The SSB protein VirE2. VirE2 is one of the most abundant Vir proteins in *vir*-induced cells (52, 92). VirE2 has a molecular mass of 60.5 kDa; it binds cooperatively to single-stranded DNA and covers the whole length of the T-DNA (Fig. 4) (51–53, 65, 104, 327). It does not complement *E. coli ssb* mutants, suggesting that it does not play a role in the replication of the Ti plasmid but, rather, functions in the processing and translocation of T-DNA (51). Several observations support this idea. Electron-microscopic analysis of VirE2-DNA complexes shows that the complexes are extremely thin (diameter, about 2 nm) and have a calculated length of about 0.18 nm per nucleotide. In comparison, the protein-DNA complex of an SSB protein like gp32 of phage T4 is 7 nm wide and 0.46 nm long per nucleotide (70), and the SSB protein of *E. coli* condenses the DNA rather than extending it (53). The dimensions of the VirE2–T-DNA complex may be a suitable transport form for the translocation of T-DNA from the bacterium into the plant cell (Fig. 4) (53). Mutants with mutations in the *virE* gene have an attenuated virulence and can be complemented by a helper strain in mixed infections, indicating that VirE2 is a diffusible product which is exported (216). The helper strains must produce not only VirE2 protein but also the products of the *virA*, *virB*, *virD*, and *virG* operons. Because VirD and VirB proteins are probably part of a transmembrane structure for T-DNA transport (see below), it is suggested that VirE2 is exported via the same transport structure. VirE2 has indeed been found in the membrane and the periplasmic fractions of induced bacteria (51, 92), although the DNA sequence does not indicate the presence of a hydrophobic signal sequence (193, 327). Thus, it is speculated that VirE2 is translocated across the membrane by specific channels (53). VirE2 may be responsible for the appropriate dimensions of the T-DNA transport form and also for the recognition of specific membrane pores, and it may guide the T-DNA into the nucleus of the plant cell (55). In addition, the protein protects single-stranded DNA against nucleolytic attack (51).

***vir*-induced membrane proteins for T-DNA translocation.** Although our understanding of T-DNA processing has steadily increased during the last few years, little is known about the translocation mechanism and machinery.

T-DNA translocation requires a direct contact between the donor bacterial cell and the plant recipient cell. Several chromosomal genes which are involved in the virulence of *A. tumefaciens*, especially in the attachment process, have been identified. The products of these genes affect the composition of exopolysaccharides. The genes *chvA*, *chvB*, and *exoC* are necessary for the synthesis of a β-1,2 glucan (76, 77, 229). Mutants with mutations in the gene *pscA*, which are not able to induce crown gall tumors, produce only little if any exopolysaccharide (292).

The VirD4 protein may be a component of the transmembrane translocation apparatus (215). The VirD4 protein, consisting of 656 amino acids, is essential for tumorigenesis (142). The protein is located in the inner membrane of induced *A. tumefaciens*; it is anchored there with the N terminus and protrudes into the periplasm, as shown by its degradation with proteinase (215). Fractionation of disrupted cells on sucrose gradients results in two different fractions containing VirD4. One of them contains VirD4 associated with fragments of the inner membrane. The other VirD4-containing fraction has a higher density. There are two explanations for the appearance of this second band: (i) the band may consist of VirD4 protein associated with adhesion sites between the inner and outer membranes, or (ii) the band may consist of VirD4 associated with other Vir proteins or host factors. Data in favor of one of these explanations are not yet available. A similar observation has been reported for the TraD protein involved in F-DNA translocation (3) (see above).

Beside the VirD4 protein, other proteins possibly constituting the transmembrane translocation apparatus are the various gene products of the *virB* operon (Fig. 4) (for a review, see reference 334). The nucleotide sequence of the *virB* operon contains 11 ORFs (158, 263, 313, 314). All proteins have been identified in *E. coli*, and the sequences indicate that nine of the VirB proteins contain either membrane-spanning regions (VirB2, VirB3, and VirB6) or a signal peptide sequence (VirB1, VirB5, VirB7, and VirB9) or at least a short N-terminal hydrophobic region which may allow association with the inner membrane (VirB8 and VirB10). VirB10 has been shown to be associated with the inner membrane of *vir*-induced cells (92, 315). The protein, which has an apparent molecular mass of 48 kDa, is probably anchored in the inner membrane by its N terminus, and a significant part of the protein reaches into the periplasm, as shown by *phoA* fusions. To obtain more details on how VirB10 is integrated in the cell membrane, cells were treated with a protein cross-linking reagent, which can cross the outer membrane to reach the periplasmic space but is unable to penetrate the inner membrane. High-molecular-mass cross-linking products (80, 115, and 140 kDa) were identified after immunoblotting. It was not clear whether these aggregates were homomultimers or heteromultimers of VirB10 and other proteins. The most abundant aggregate was the 140-kDa complex, which could be a trimer of VirB10. The fact that VirB10 is essential for tumor formation, together with the identification of a VirB10 protein aggregate spanning the membrane, suggests that VirB10 may constitute a structural component of the T-DNA translocation apparatus (315, 316).

The proteins VirB4 and VirB11 have no typical signal peptide or any membrane-spanning regions. Nevertheless, they are associated with the inner membrane (92). The hydrophilic VirB11 protein is essential for tumorigenesis and has a molecular mass of 38 kDa (313, 315). VirB11 was overproduced in *E. coli* and purified (50). In agreement with the prediction of a type A mononucleotide-binding site (309) as deduced from the DNA sequence, the purified protein binds and hydrolyzes ATP. The ATPase activity is not stimulated by various DNA effectors such as calf thymus DNA, single-stranded or double-stranded oligonucleotides, or linear or circular plasmid DNA, and it is also not stimulated by border sequences or T-DNA. Mononucleotide-binding sites have also been identified in the sequences of VirB4 and VirB5. It is possible that ATP hydrolysis by one or several of these proteins provides the energy for T-DNA translocation. An intact nucleoside-triphosphate-binding domain of VirB4 is essential for the virulence of *A. tumefaciens* (30). The purified VirB11 protein shows autophosphorylation in the presence of ATP in vitro. It is proposed that autophosphorylation may change the conformation of the protein, allowing the interaction with components of the T-DNA translocation apparatus (50). If VirB11 revealed kinase activity on other proteins, the phosphorylation of those

proteins may activate them to form the apparatus for DNA translocation.

In studies to identify the subcellular localization of seven of the VirB proteins (VirB1, VirB4, VirB5, VirB8, VirB9, VirB10, and VirB11), six of them (not VirB5) were found in both the inner and outer membranes, leading to the interpretation that a complex channel spanning both membranes may exist. Such a channel could be located at the zones of adhesion between the two membranes. This would imply that the DNA translocation follows a one-step mechanism in which both membranes are passed at once (293) (Fig. 4).

T-DNA translocation has been compared to bacterial conjugation (for reviews, see references 333 and 335). There are indeed several similarities between these two processes, and T-DNA transfer can perhaps be regarded as an adaptation of the conjugation process to allow transfer of DNA from a bacterial cell to a plant cell. Similarities in structure and functions of the proteins are discussed below. Besides having analogous functions in the DNA-processing events (46, 317), the translocation machineries appear to be very similar (compare Fig. 3 and 4 and Table 1). Thus, it was demonstrated that VirB and other Vir proteins direct conjugational translocation of the Ti plasmid between *Agrobacterium* strains (280) and that VirB and VirD4 can replace the analogous *tra* gene products during translocation of a nonconjugative but mobilizable IncQ plasmid between agrobacteria (28). Further support for a conjugative translocation model of T-DNA came from the observation of striking sequence homologies between VirB2 and TraA, encoded by the F factor (262). Additionally, the two proteins are almost identical in size, are similarly processed to a mature form, and are located in both the inner and outer membranes. Since the processed TraA protein, the pilin, is the main component of the F pilus, it has been speculated that a conjugative pilus might also be involved in T-DNA translocation (262).

DNA UPTAKE DURING NATURAL TRANSFORMATION

Genetic transformation is a process by which a bacterial cell takes up free DNA from the surrounding medium, incorporates it, and expresses the newly acquired trait. Transformation was the first mechanism of genetic exchange reported in bacteria (111). Within a variety of gram-positive and gram-negative bacteria, it is part of the normal physiology to become competent, i.e., to be able to take up DNA. The transformation process itself may be divided into separate steps which are common to all bacteria. These steps are (i) development of competence; (ii) binding of DNA; (iii) processing and uptake of DNA; and (iv) integration of DNA into the chromosome by recombination and expression. There are several reviews which deal with the subject of natural competence and transformation (163, 164, 183, 272, 282, 283, 306).

Natural Transformation in Gram-Positive Bacteria

The best-studied gram-positive species with respect to natural transformation are *Streptococcus pneumoniae* and *Bacillus subtilis*, and several reviews, most of them on *B. subtilis*, have been published (78–82). The map of competence genes is probably not complete, but a lot of the genes involved in the transformation process have been identified and sequenced, and studies concerning the mechanism and machinery of DNA translocation are in progress.

Competent cells of *B. subtilis* and *S. pneumoniae* bind double-stranded DNA noncovalently (204) and without base sequence specificity. The precise location of the binding sites

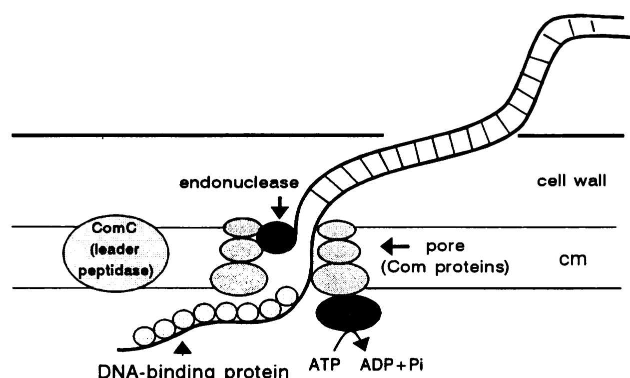

FIG. 5. DNA uptake during transformation of gram-positive bacteria. The transmembrane channel is probably formed by ComG and ComE proteins. Some of the ComG proteins may be processed by the leader peptidase ComC. During the passage across the cytoplasmic membrane (cm), one strand of the double-stranded donor DNA is degraded by a membrane-bound endonuclease.

on the cell surface is not known, and a receptor for DNA at the cell surface has not yet been identified. For *S. pneumoniae* it was proposed that the receptor is a membrane protein stabilized by polysaccharide components of the cell wall and has to be unmasked by an activator protein during competence development (259). The number of DNA-binding sites has been estimated to be about 50 per cell, and a large DNA molecule may be associated with several binding sites (83, 163, 164, 266). Binding of the DNA is accompanied by nicking of the DNA with a frequency of about one break per 2×10^6 Da of single-stranded DNA (165, 166). A competence-specific endonuclease has in fact been identified in the membrane of *S. pneumoniae* (99). After binding, donor DNA is rapidly further processed by the introduction of double-strand breaks (15, 83). By about 1 to 2 min after the addition of DNA to competent cells, the DNA becomes resistant to added DNase I (179) and is taken up by the cells with a linear kinetics (284, 285). The DNA taken up by the cells can be isolated in the form of single-stranded fragments with an average length of about 10 kb (202, 224). In the cell this single-stranded DNA is coated by a competence-specific SSB protein, possibly as a protection against nucleases (90, 205) (Fig. 5). The appearance of single-stranded DNA is accompanied by the release of acid-soluble nucleotides into the medium in amounts which indicate that one strand is completely degraded (67, 83, 143). The polarity of DNA entry in *B. subtilis* is not yet clear; however, in *S. pneumoniae* the uptake of the single-stranded DNA starts with the 3′ end (195, 304).

Late competence genes and their products. Gene products which are necessary for transformation are classified into two groups. The first group of early competence gene products plays mainly a regulatory role during competence development. The second group of late competence proteins is required for binding, processing of DNA, and assembly of the DNA translocation machinery; in *B. subtilis* it contains the competence-specific SSB protein, the entry nuclease, and the products of the gene *comC* and of the operons *comE*, *comG*, and *comF*.

(i) Endonuclease involved in the entry of donor DNA. In 1962 Lacks already realized the importance of the major endonuclease (endonuclease I) of *S. pneumoniae* for the entry of DNA during transformation (162). He proposed that the endonuclease hydrolyzes one strand and that without further

requirement for energy the remaining intact single strand will be pulled into the cell. Therefore, the endonuclease was termed translocase. Several mutants with mutations in the gene for the *S. pneumoniae* endonuclease were tested for their ability to be transformed. In *end-1* mutants the endonuclease activity was reduced to 10% but the transformation was not impaired. However, in a second class, the *noz* mutants, the endonuclease activity was reduced to below 1% and transformation longer occurred (167, 168). The activity of *S. pneumoniae* endonuclease I in cell extracts is detected only after treatment with detergents, indicating that the enzyme is located in the cell membrane (169). The *endA* gene product is a 30-kDa protein with a typical N-terminal hydrophobic sequence which anchors the protein in the membrane (230). Plasmids with the *endA* gene can complement *endA* mutants by restoring their transformability. In gel filtration experiments endonuclease I was found in a large complex with a molecular mass of about 250,000 Da (243). The nature of this complex was not clear. It dissociated only after proteolytic degradation. The presence of a mild detergent was necessary during purification of the complex to maintain its nuclease activity. Therefore, it seems that the complex is composed of hydrophobic proteins. This led to the model of a membrane-integrated doughnut-like structure with an aqueous pore in the center which would allow the passage of the transforming DNA single strand while the complementary strand is degraded by the asymmetrically attached endonuclease I (243) (Fig. 5). This model has not been challenged since then.

DNA entry-deficient mutants of *B. subtilis* have also been isolated, and the same correlation between the extent of DNA breakdown and DNA entry as in *S. pneumoniae* was shown to occur in *B. subtilis*, suggesting that in both strains an endonuclease is involved in DNA entry (207, 208). Although a membrane-bound enzyme complex involved in DNA translocation in *B. subtilis* has been described, the data are somewhat confusing. The enzyme complex with a molecular mass of 75 kDa consists of four subunits, two of 17 kDa and two of 18 kDa (268–270). The corresponding genes *comI* and *comJ* have been cloned and sequenced (308). The 17-kDa protein (ComI) has endonuclease activity against double-stranded DNA and may represent the analog of the *S. pneumoniae* endonuclease I (270, 271). Some mutants with deficiencies in *comI* and *comJ* have a residual transformation efficiency of only 5% (308), but other mutants with null mutations in the *comI* gene exhibit normal transformability, which raises doubts that the 17-kDa protein is the entry nuclease at all (cited in reference 81). In earlier publications, mutants with mutations in the 18-kDa protein (ComJ) were reported to no longer bind donor DNA (268). In subsequent experiments a *comJ* insertion mutant showed wild-type DNA-binding activity (308). A function of the 18-kDa protein as modulator of the nuclease was considered by the authors. Further experiments are necessary to clarify the question whether the ComI-ComJ complex is the uptake nuclease analogous to *S. pneumoniae* endonuclease I.

(ii) Proteins of the DNA translocation apparatus. The late competence genes or operons of *B. subtilis*, which may be involved in the building up of a DNA translocation apparatus, were identified by Tn*917lac* mutagenesis and include the loci *comC*, *comE*, *comF*, and *comG* (Fig. 5) (6–8, 116, 182). Mutants with mutations in the *comC* gene are deficient in binding and uptake of donor DNA. ComC is a hydrophobic protein of 258 amino acids (200) and has homology to the products of the genes *xcpA* of *Pseudomonas aeruginosa* and *pulO* of *Klebsiella oxytoca*. The last two gene products are leader peptidases processing the prepilin proteins to the mature form involved in protein secretion (213, 228). In *E. coli* expressing *comC* and *pilE*, the gene for the prepilin of *P. aeruginosa*, the prepilin is processed to the mature form, suggesting that ComC is a leader peptidase for the processing of some components of the DNA translocation apparatus (86). Mutants defective for *comG* have the same phenotype as *comC* mutants because they cannot bind DNA and are completely competence deficient (115). The *comG* locus is an operon consisting of seven ORFs (7). With the exception of ORF1, which may encode a primarily hydrophilic protein of 356 amino acid residues, all other hypothetical polypeptides of the *comG* operon have a hydrophobic character, with at least one potential membrane-spanning region. Mutants with mutations in *comG1* had a resolution-negative phenotype of competent and noncompetent cells on a Renografin gradient. The hypothetical protein shows significant sequence similarity to the *virB11* gene product of *A. tumefaciens* involved in T-DNA translocation. Like VirB11, the hypothetical ComG1 protein may have a nucleoside triphosphate-binding site consisting of type A and type B domains (309) (Fig. 5). As well as its possible role in DNA translocation, the ComG1 protein plays a regulatory role because it is required for the full expression of *comE*. Only the ComG3 protein was further characterized with respect to its localization in the cell (42). The solubility of ComG3 in alkaline solution, which differs depending on the presence or absence of other competence proteins, suggests that a specific organization with other Com proteins occurs in the membrane. The N-terminal part of the small proteins ComG3, ComG4, and ComG5 exhibit homology to a class of pilin proteins from *Bacteroides*, *Pseudomonas*, *Neisseria*, and *Moraxella* species (125). The proteins also show homology to each other, implying that they have a similar function. The homology to the pilins, which assemble a helical structure with a central pore, may be taken as an indication that the ComG proteins may build up a transmembrane channel for DNA passage (Fig. 5). Proteins ComG3 to ComG5 may be the substrate for the leader peptidase ComC (125). However, this is entirely speculative, and the existence of such a transmembrane structure has not yet been demonstrated.

Recently, two further late competence operons, the *comF* locus and the *comE* locus, have been sequenced (116, 182). The *comF* operon contains three ORFs (182). Disruptions of these ORFs reduce the transformability of *B. subtilis*; disruption of ORF1 does so more drastically than disruption of ORF2 and ORF3. The hypothetical protein of ORF1 belongs to the family of ATP-dependent RNA-DNA helicases with an extensive similarity to the PriA (formerly n′; a component of the primosome) protein of *E. coli*. The ORF1 protein contains at most one single possible membrane-spanning region, and preliminary experiments indicate a membrane association of the protein. From the similarity to the PriA protein, which is involved in primosome assembly, we can conclude that the ORF1 product may also interact with other proteins. Hahn et al. therefore propose that ORF1 may encode an ATP-dependent DNA translocase, which is located within a membrane-associated DNA uptake machinery driving the single-stranded DNA into the cell. The *comE* operon contains three ORFs in one orientation and one in the other orientation (116). The makeup of the amino acid sequences deduced from the nucleotide sequences suggests that two of the hypothetical gene products are integral membrane proteins (ORF1 and ORF3). ComE1 has only one predicted transmembrane region near the N terminus and seems to be involved in DNA binding at the cell surface. ComE3 reveals 8 to 10 transmembrane segments and appears to be required for DNA translocation. Hahn et al. suggest that ComE3 functioning as a polytopic

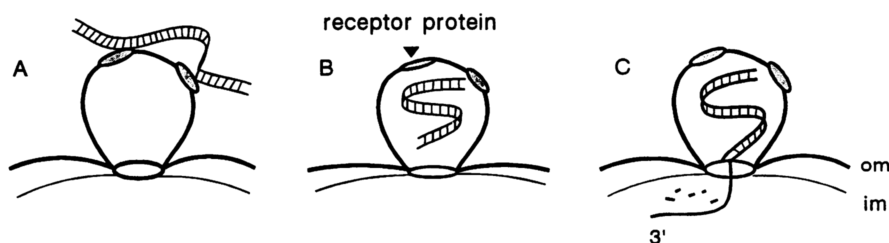

FIG. 6. Transformasome-mediated uptake of DNA during transformation of *H. influenzae* (147). (A and B) Double-stranded donor DNA is bound sequence specifically (A) and is taken up into the transformasomes (B) by an unknown mechanism. (C) The DNA transfer into the cytoplasm is linked to the degradation of one strand as in gram-positive bacteria. Abbreviations: om, outer membrane; im, inner membrane.

membrane protein is an excellent candidate for a water-filled pore for DNA translocation.

Little is known about the late competence genes and their products in *S. pneumoniae*. Of 14 competence-induced proteins, 11 have been localized in cells, but they have not yet been assigned to their genes. Some of the proteins were found in the cytoplasmic membrane and are candidates for the DNA translocation apparatus (203, 307). Only two of the competence-specific proteins have been partially characterized so far, an SSB protein (307) and the *comA* gene product, possibly a transport protein for the export of the competence factor, a protein which is necessary for the coordination of competence induction among the cells of a culture (135).

PHB—a channel for DNA transfer? Although the hypothesis of DNA transport mediated by a channel consisting of membrane proteins predominates, there is accumulating evidence for an interesting alternative, namely for the nonprotein nature of the channel. It was observed that during competence development of bacteria the concentration of polyhydroxybutyrate (PHB), a ubiquitous constituent of cells, increases, e.g., by a factor of 50 in *Azotobacter vinelandii*, by a factor of 10 in *B. subtilis*, and from a nondetectable amount to 1.1 $\mu g/10^{10}$ cells in *Haemophilus influenzae* (235). Part of the PHB is found in the cytoplasmic membrane. Electron-microscopic studies suggest significant alterations of the membrane structure compared with that in cells in the noncompetent state (234). The PHB in the membrane of natural competent bacteria and of *E. coli* cells treated with Ca^{2+} is complexed with Ca^{2+} and polyphosphate. The amounts of the complex are smaller in natural competent cells than in Ca^{2+}-treated *E. coli* cells. Thus, for studies concerning its structure and function, the complex was isolated from Ca^{2+}-treated *E. coli* cells (236). After extraction of the complex, the amounts of PHB, polyphosphate, and Ca^{2+} were assayed and the lengths of the PHB chains (120 to 200 subunits) and polyphosphate chains (130 to 170 subunits) were determined by viscosity measurements and size exclusion chromatography. From these data it was concluded that the complex contains PHB, polyphosphate, and Ca^{2+} in molar ratios of 1:1:0.5. A molecular model for the complex proposes a helix with a lipophilic exterior formed by PHB which is stabilized in the interior by Ca^{2+} and polyphosphate, resulting in a structure with a diameter of 2.4 nm, a helical rise of 0.4 nm, and a length of 4.5 nm. Since single-stranded DNA itself is a polyphosphate and resembles the polyphosphate chain in the complex, it was proposed that DNA may be transferred through the PHB cylinder, whose inner diameter is large enough to allow the passage of single-stranded DNA but not of double-stranded DNA (236). It is postulated that after binding of the DNA to the cytoplasmic membrane, the polyphosphate of the complex is drawn into the cytoplasm, closely followed by the single-stranded DNA (233). Reusch and Sadoff do not discuss the obvious disagreement with the fact that Ca^{2+}-treated *E. coli* cells are equally well transformed by double-stranded and single-stranded DNA. PHB, the PHB complex, and its possible functions were reviewed by Reusch (233). The crucial experiment whether mutants unable to synthesize PHB can be transformed has not been performed.

Natural Transformation in Gram-Negative Bacteria

Natural transformation has been observed with a number of gram-negative bacteria such as *Haemophilus*, *Acinetobacter*, *Neisseria*, and *Moraxella* spp. New strains capable of transformation are continuously being found. The best-studied gram-negative organism with regard to transformation is the genus *Haemophilus* (for reviews, see references 107 and 148). In contrast to the examples of *B. subtilis* and *S. pneumoniae*, *Haemophilus* spp. bind and take up only homospecific DNA, a characteristic also found, e.g., in *Neisseria* spp. (108, 109). However, a more interesting feature with respect to the DNA translocation mechanism is the presence of specialized membrane structures, the transformasomes, involved in this process.

Transformasomes—specific membrane structures in *Haemophilus* species. Early biochemical and immunological studies have shown that there are extensive rearrangements in the outer and inner membranes of *Haemophilus* spp. during the development of competence (31, 62, 145, 337, 338). Morphological studies by electron microscopy demonstrated specific membrane extensions on the surface of *H. influenzae* and *H. parainfluenzae* which were termed transformasomes. These structures extend about 35 nm from the cell surface and have a diameter of about 20 nm. About 10 to 12 transformasomes are found per cell. They are located at adhesion sites between outer and inner membranes and are composed mainly of outer membrane components (63, 147). After incubation of competent cells with transforming DNA, structurally intact transformasomes can be isolated by extraction with organic solvents and are shown to contain double-stranded DNA (146). In the transformasomes the DNA is protected against externally added DNases and also against cellular restriction endonucleases. In *H. influenzae* the transformasomes remain at the cell surface after addition of DNA, whereas in *H. parainfluenzae* they are translocated into the periplasm (17). Linear DNA is very rapidly transferred from the transformasomes into the cell, and this is accompanied by partial degradation. Circular DNA or DNA with hairpin structures at the ends remains within the transformasomes. In accordance with these findings, the following model for *Haemophilus* transformation was proposed (146) (Fig. 6). Linear double-stranded homologous DNA binds to a receptor protein at the surface of the

transformasomes via an 11-bp specific DNA uptake sequence (64, 69) and is then translocated into the transformasome by an unknown mechanism. In this state the DNA is protected against nucleases. The DNA with free double-stranded ends can exit the transformasome into the cytoplasm through an opening of about 5 nm, which can be detected in the electron microscope (146). During passage of the cytoplasmic membrane, one strand of the DNA may be degraded while the complementary strand enters the cytoplasm with the 3′ end ahead and is incorporated into the chromosome by recombination (18). The 5′ strand may be degraded by a nuclease, as described for gram-positive bacteria (243). Although degradation of donor DNA was observed, the conversion of double-stranded to single-stranded DNA has not yet been shown for *Haemophilus* spp. but has been reported for the gram-negative species *Acinetobacter calcoaceticus* (218).

Genes and gene products involved in DNA uptake. Numerous transformation-deficient mutants of *H. influenzae* have been isolated, but their characterization on the molecular level is still in its early stages (19, 63, 211, 295, 296). The gene product of *rec-2* is involved in DNA translocation (19, 173, 211). Mutants with transposon insertions in the *rec-2* gene fail, among other defects, to translocate DNA from the transformasomes into the cell. The transforming DNA remains in the transformasome even 1 h after addition, and less than 3% of the input DNA is found as acid-soluble material in comparison with 15 to 25% with wild-type cells (173). The *rec-2* defect is complemented by the cloned *rec-2* locus (173, 194). Another gene essential for transformation of *Haemophilus* spp. is the *com-101A* gene. Like the *rec-2* mutants, the *com-101A* mutants are defective in the processing of the donor DNA (173). The competence-specific ComA-101A protein has a molecular mass of 26.5 kDa and a pI of 10.3, indicating that it may interact with DNA (172). Both the *rec-2* and the *com-101A* gene products are good candidates for the nuclease involved in processing of DNA during transfer from the transformasomes into the cytoplasm of the cell. However, all attempts to demonstrate nuclease activity of the gene products obtained by overexpression in *E. coli* failed (173). The product of *com-101A* exhibits a striking 22% homology to the product of *comF* ORF3 of *B. subtilis* (172), and the product of *rec-2* has a 24% homology to the product of *comE* ORF3 (116). This indicates that despite the differences in the cell surface of gram-negative and gram-positive organisms and the differences in the early steps of the transformation processes of *Haemophilus* spp. and *B. subtilis*, the DNA translocation machineries are very similar.

Sequence homologies were found not only between gene products involved in transformation of *Haemophilus* spp. and *B. subtilis* but also between other transport systems. The gene product of ORFE which is involved in *Haemophilus* transformation is homologous to gpIV of phage fd and PulD of *Klebsiella pneumoniae* (296). Gene product IV of phage fd is involved in phage assembly and secretion of phage particles and may form an exit for the assembling phage (for a review, see reference 245). PulD is part of the pullulanase secretion apparatus (71). Thus, these proteins may be members of a class of proteins involved in the transport of macromolecules across bacterial membranes (296).

A further protein involved in the transformation of *Haemophilus* spp. is a periplasmic oxidoreductase, the Por protein (294). Mutants with mutations in the *por* gene are deficient in binding and uptake of DNA. In competent cells, several proteins are displaced from the inner membrane into the outer membrane. This displacement is not observed in *por* mutants. It is proposed that the disulfide oxidoreductase is required for correct assembly and/or folding of one or more proteins with disulfide bridges in the cell envelope essential for competence development or DNA binding and uptake. Additionally, Tomb (294) demonstrated by Southwestern (DNA-protein) analysis that several competence-specific proteins of the outer membrane have affinity to double-stranded DNA and that a 32-kDa protein binds to single-stranded DNA. These proteins may be components of the DNA-binding and DNA uptake apparatus. Further investigation of genes and gene products is necessary to elucidate the mechanism of DNA uptake via transformasomes, which is unique insofar as it includes translocation of double-stranded DNA into the transformasomes and of single-stranded DNA from the transformasomes into the cytoplasm.

So far for gram-negative bacteria, no proteins involved in a DNA uptake machinery have been characterized and no membrane-bound nuclease has been identified. Nonetheless, it is assumed that DNA translocation occurs with the help of specialized pores or channels, as required for all DNA translocation processes (148).

NEISSERIA BLEBS—NEW MECHANISM OF INTERCELLULAR PLASMID TRANSFER?

Another biological process in which DNA possibly has to traverse the bacterial envelope has been detected recently in *Neisseria gonorrhoeae*. During growth, *N. gonorrhoeae* forms and releases specialized DNA-containing membrane vesicles, which are termed blebs (73, 75). DNA-containing blebs have been detected so far in 14 gram-negative strains (74). Two different bleb fractions can be isolated from the culture medium of *N. gonorrhoeae*, the BI fraction, which seems to originate from cytoplasmic membrane-derived vesicles, and the BII fraction, which contains lipopolysaccharides and proteins from the outer membrane. Both types of blebs contain RNA and linear and circular DNA, but only in the BII blebs is the DNA protected against DNase I digestion. The presence of outer membrane material and the protected stage of DNA is reminiscent of the transformasomes of *H. influenzae*; however, in contrast to these, the blebs seem to mediate plasmid transfer from one cell to another. Incubation of wild-type gonococci with blebs from antibiotic-resistant strains resulted in acquisition of the R plasmids, indicating intercellular plasmid transfer. Transfer of chromosomal markers has not been observed. Until now, nothing was known about the mechanism of DNA transfer with the help of blebs. It is not clear whether a translocation of DNA across bacterial membranes is necessary during this process. Thus, it is possible that the blebs are produced at the cell surface and that DNA is translocated across the membranes into the blebs. After association of the blebs with a recipient cell, translocation of DNA across the membranes of the bleb and the recipient cell may be necessary. Alternatively, formation of the blebs may be a process like the separation of minicells from certain *E. coli* strains. The DNA could reach the cytoplasm of the recipient by fusion of the bleb membrane with the membrane of the recipient cell. The latter mechanisms would not include DNA translocation across membranes.

COMPARISON OF DNA TRANSLOCATION MECHANISMS

There are several important differences in the natural DNA translocation processes which suggest that they follow separate mechanisms and therefore have unique characteristics. A main point is that the transferred DNA is either single stranded or double stranded. In the cell, single stranded DNA is mainly covered by SSB protein (198). Thus, DNA can be transferred

TABLE 1. Homologies between proteins involved in various DNA translocation processes

Structural feature	Transformation protein secretion pilation	Reference(s)	T-DNA transfer conjugation protein secretion	Reference(s)
Translocation apparatus	ComG3, 4, 5, 6	7	VirB2, 3, 4, 5, 10	263
	PulG, H, I, J	81, 227	TrbC, D, E, F, I ↔ TraA	175, 262
	PilE	81, 321	PtlB, C, D	261, 320
Leader peptidases (cytoplasmic membrane)	ComC	200	TraQ	328
	PulO	86		
	PilD	213		
Nucleotide-binding proteins (membrane associated)	ComG1	7	VirB11	7, 50, 85
	PulE	71, 81 ↔	TrbB	175, 206
	PilC	81, 321	PtlH	320
			VirB4	316
			TrbE	175
Pilot proteins			VirD2	142
			TraI (RP4)	223

in a complex with SSB protein, as shown for T-DNA with VirE2 or not as in the case of the F plasmid. Association with specific proteins influences the dimension and the charge of the DNA to be translocated. A further difference is the direction of the translocation. The DNA is transferred from the inside of the cell to the outside during T-DNA transfer and conjugative transfer from the donor cell and is transferred from the outside of the cell to the inside during phage infection, transformation, and conjugative transfer into the receptor cell. Finally, there are essential differences in the bacterial envelopes which have to be crossed (gram negative and gram positive).

Nevertheless, there are some structural and functional features which seem to be common to DNA translocation mechanisms; data concerning amino acid sequence homologies within the group of proteins involved in translocation across bacterial membranes are accumulating. It was rather surprising to find that the homologies were not confined to the proteins of DNA translocation but were also identified in proteins involved in the secretion of proteins and in the assembly of some pili (see, e.g., references 125, 227, and 321). The considerations about homologies are limited mainly to the processes of transformation, conjugation, and T-DNA transfer, but there are at least some analogous proteins and structures involved in infection by bacteriophages. The points discussed below, which will focus on the similarities between the translocation mechanisms, are summarized in Table 1 and Fig. 7. The corresponding references may be found in Table 1.

DNA Translocation Apparatus

The most striking similarities are found in the proteins making up the DNA translocation apparatus of various systems (Table 1). A complex channel composed of several proteins is probably built up for DNA translocation during transformation (7, 42, 81), conjugation, and T-DNA transfer (262, 293) (Fig. 3 to 5). A similar channel was proposed for the secretion of several proteins, for example the pullulanase (Pul proteins [226]) of *K. oxytoca* and the protein toxin of *Bordetella pertussis* (Ptl proteins [320]). A further example involves the pili of *P. aeruginosa*, which are built up by the pilin subunits (Pil proteins [321]) in a helical structure with a central pore (98). All these proteins which form a channel or a pilus-like structure ("pseudopilus" [227]) share extensive homology, especially five of the VirB proteins with five of the Trb proteins of RP4 and three of the Ptl proteins, and four of the ComG proteins with the Pil and Pul proteins, respectively. No homologies between the VirB and the ComG proteins have been described. Therefore, Table 1 is divided into two columns. An analogous structure to the complex DNA translocation channel of Tra, Com, and Vir proteins may be the phage tail of phage T5, composed of the pore-forming protein pb2, which is able to span the hole bacterial envelope (Fig. 1B).

Leader Peptidases

Some of the proteins involved in channel formation are located in the periplasm; this means that they have to be processed by a leader peptidase. It was demonstrated that ComC was able to process the prepilin of *Pseudomonas* spp. (prePilE [86]). Additionally, ComC has homologies to the leader peptidases PulO and PilD. A further leader peptidase

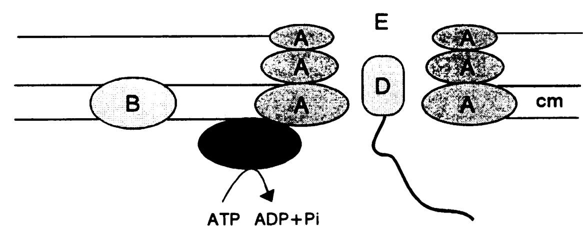

FIG. 7. Common components in the DNA translocation apparatus during conjugation, T-DNA transfer, and transformation. A, Transmembrane channel formed by pilin-like proteins; B, membrane-located leader peptidase for the processing of pilin-like proteins; C, membrane-associated nucleotide-binding protein; D, pilot protein; E, specific polarity of DNA transfer. Abbreviation: cm, cytoplasmic membrane.

may be TraQ of F (328). Expression of the *traQ* gene was a prerequisite for production of large amounts of mature pilin. Prepilin produced from a plasmid with *traA* was processed effectively only when a second plasmid with the *traQ* gene was in the cell. No homologies of TraQ to other leader peptidases have been described.

Nucleotide-Binding Proteins

In all translocation systems, at least one protein with a consensus sequence for a nucleotide-binding site has been identified. Proteins VirB11, TrbB of RP4, and ComG.1, involved in DNA translocation, and PilB and PulE, involved in pilus assembly and protein secretion, respectively, share homology to each other (indicated by the arrows in Table 1). They are all hydrophilic proteins, which may be associated with the cytoplasmic membrane by interactions with membrane proteins. The significance of the nucleotide binding is not known for any of the proteins. It is speculated that hydrolysis of the nucleoside triphosphate provides energy for the assembly of the translocation apparatus or for the DNA translocation process itself.

Pilot Proteins

DNA is probably never translocated in a naked form but is always associated with proteins. This rule is true not only for prokaryotes but also for eukaryotes (for a review, see reference 54). The DNA-binding proteins play a crucial role in DNA translocation and may have different functions. One function may be that of a pilot protein which is bound to the ends of DNA and facilitates translocation across the membranes (155). It is not defined whether the pilot protein is responsible for the DNA translocation itself and/or for opening or perhaps closing of the channel. In all DNA translocation processes with the exception of transformation, a protein was assumed to have a pilot function, e.g., gp2 of phage T4, VirD2 of the Ti plasmid, and TraI of RP4. A pilot protein has also been postulated for the transformation process (166). This protein may bind to the DNA after nicking at the cell surface. Such a protein has not yet been identified. VirD2 and TraI share some sequence homologies. Further DNA-associated proteins such as SSBs do not seem to be common in all systems.

Polarity of DNA Translocation

In the various systems, DNA seems to be translocated always with the same end ahead. This leading end may be determined by the polarity of single-stranded DNA or perhaps by the pilot protein. T-DNA, RP4, and F are translocated as single strands with the 5′ end ahead. Uptake of DNA by competent *Streptococcus* spp. occurs with the 3′ end ahead. Also, in the case of infection by bacteriophages with double-stranded DNA (e.g., T5), the translocation begins with the same end.

CONCLUDING REMARKS

In this review, data on the structural and functional requirements for DNA translocation processes across bacterial membranes during phage infection, conjugation, T-DNA transfer, and transformation have been summarized. During the last few years a lot of genes involved in DNA translocation have been identified and sequenced, and their gene products have been isolated and at least partially characterized. From these data it can be concluded that DNA translocation probably occurs through a protein transmembrane channel. Additionally, the findings of striking functional and structural similarities in the natural DNA translocation processes, especially during conjugation, T-DNA transfer, and transformation, converge to a common model for DNA translocation. A transmembrane channel which may span the whole bacterial envelope is built up by several different pilin-like proteins, some of which are processed by a leader peptidase. A pilot protein bound to the first end of DNA to be translocated guides the DNA through the channel. Energy for the assembly of the DNA translocation apparatus or the translocation process is provided by ATP hydrolysis of a membrane-associated nucleotide-binding protein. Until now the hypothesis of channel-mediated DNA transfer has rested mainly on genetic data and the finding that many of the gene products involved in DNA translocation are located in the membrane. There are only two examples of pore-forming proteins which are part of the injection apparatus of bacteriophages T5 (pb2) and fd (gp3). Thus, it would be necessary to intensify electrophysiological experiments with the aim of reconstituting a channel and showing the passage of DNA. Recent advance in the methods of physicochemical characterization of channel proteins such as patch clamp and voltage clamp analyses will certainly stimulate further studies to verify the hypothesis of channel-mediated DNA translocation.

Evident similarities between the different DNA translocation systems suggest a common evolutionary origin of the assembly, structural subunits, and the structure of a DNA transfer apparatus. The similarities are not only confined to processes of DNA translocation but may also be extended to the excretion of several proteins. The excretion apparatus, like the DNA translocation apparatus, consists of a transmembrane channel. The components of this channel are processed by a leader peptidase, and a nucleotide-binding protein probably energizes the assembly of the channel or the excretion process. Thus, it can be speculated that the processes of transport of macromolecules (nucleic acids and proteins) may have a common evolutionary history.

In this review, the translocations of DNA during phage infection, conjugation, T-DNA transfer, and transformation have been discussed. It should be mentioned that these natural processes are probably not the only ones in which DNA translocation occurs. There are further examples of cases when nucleic acids leave or enter a bacterial cell. (i) A recently detected mechanism of DNA transfer may be the bleb formation of *N. gonorrhoeae* and some other gram-negative bacteria. (ii) Natural transformable bacteria release DNA into the medium. This DNA release is not caused by lysis of the cells but may be a form of excretion. (iii) The periplasmic enzyme endonuclease I of *E. coli* is associated with RNA when isolated from the periplasmic space, indicating that RNA is present in the periplasmic space. This means that RNA has to traverse the cytoplasmic membrane either in a free form or complexed with endonuclease I. (iv) As filamentous phages are assembled at the membrane, DNA translocation processes may be involved in the morphogenesis of these phages. Until now it has not been decided whether translocation of nucleic acids across bacterial membranes is involved in these processes.

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