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REVIEW PAPER

Conjugation in Bacilli

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Abstract—The review considers experimental data on the conjugal transfer of plasmids in the *Bacillus cereus* and *Bacillus subtilis* groups (the transfer of large self-transmissible plasmids and the mobilization of small plasmids). Conjugation in bacilli is compared with conjugation in *E. coli* dependent on the F factor. Conjugation of bacilli in their natural habitats is also discussed.

Key words: conjugation, F factor, plasmid, *Bacillus*.

Conjugation in bacteria was described by J. Lederberg as far back as 1946. Since then, the number of studies devoted to this phenomenon has increased in an avalanche-like manner. Conjugation, as a type of bacterial mating, is used for the mapping of chromosomes and for the transfer of plasmids from one cell to another. Some aspects of conjugation, such as the preparation of cells for contact, the cell-to-cell contact itself, and the mechanisms by which DNA is transferred between cells, are studied not only by geneticists but also by researchers working in the field of bacterial biochemistry and physiology. Conjugation is also of interest to researchers studying microbial ecology and the horizontal transfer of plasmids and chromosome genes in the natural habitats of microorganisms [1–3]. Some aspects of conjugation are specific to bacterial genera (including *Bacillus*) and even species.

Before proceeding further, let us briefly consider conjugation in *Escherichia coli*, which is dependent on the F factor (fertility factor, which represents a sex or conjugative plasmid) and has been comprehensively studied. The results of the investigation of the F factor of *E. coli* provided the basis for nearly all later studies of conjugation in various bacteria, including conjugation in *E. coli* dependent on conjugative plasmids that differ from the F factor. Relevant information concerning conjugation in *E. coli*, which was considered in a number of reviews [4–10], will be compared with that concerning conjugation in bacilli.

1. THE STRUCTURE AND ROLE OF THE F FACTOR IN *E. COLI* CONJUGATION

The F factor is a large (about 100 kb) circular plasmid, occurring in cells in 1–2 copies. The F factor genome, which is completely sequenced, has the following functional regions: the *tra* region, whose genes are arranged in an operon responsible for cell contact and DNA transfer to a female cell; the so-called leading region, which is the first to enter the female cell; three loci with the *repFIA*, *repFIB*, and *repFIC* genes, which control replication; and three transposable elements, *Tn*1000, IS2, and IS3 (the latter in two copies). Due to their transposable elements, the F factor can integrate with other plasmids and bacterial chromosomes, forming cointegrates.

Conjugation begins when male (or donor) and female (or recipient) cells come into contact. The male, but not female, cells carry the F factor and have sex fimbriae, or sex pili, which are flexible appendages about 20 μm in length (i.e., several times longer than the cells themselves). Sex pili have the form of a hollow cylinder with an outer diameter of about 80 Å and an inner diameter of about 20 Å, made of helically packed pilin protein subunits. One cell typically has 1–2 pili (sometimes more). The intracellular ends of sex pili lie in the periplasmic space, between the inner membrane and the cell wall. Sex pili are assembled from the pilin subunits in the periplasmic space. The composition of the sex pilus tip slightly differs from that of the pilus remainder, due to which DNA-containing filamentous phages (like M13 and fd) bind to the pilus tip, whereas RNA-containing phages (like R17 and MS2) bind laterally to the remainder of the pilus. The synthesis of sex pili is controlled by 17 genes located in the *tra* region of the F factor. This region occupies no less than one-third of the whole length of the F factor and contains overall 36 genes. The sex pili whose synthesis in *E. coli* is independent of the F factor are shorter, less flexible, and are made of pilins that differ from the F-dependent pili (or F pili). The F factor–dependent conjugation is more efficient in liquid media, whereas conjugation dependent on other sex factors is more efficient on solid surfaces, such as the surface of membranes filters or agar media.

When the tip of the pilus of a male cell touches a female cell, the pilus contracts. The contraction and a cascade of subsequent events are induced by a signal generated in response to the contact of the pilus tip with

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the surface receptors of the female cell. Until the pilus contracts, the male and female cells can easily be separated by shaking. Otherwise, the female and male cells come into close contact via their walls, forming aggregates of 2 to 50 cells. These aggregates are difficult to separate. The cell wall at the site of contact becomes electron-dense, ultimately giving rise to a pore, through which DNA can pass from the donor to the recipient cell. It has long been considered that donor DNA enters recipient cells through the contracted hollow pilus, but there is not as yet any convincing evidence to confirm this. Some researchers suggest that the pilus merely serves as a harpoon to drag a recipient cell up to the donor cell, while donor DNA passes into the female cell through the pore [11]. Which of these viewpoints is true remains unknown.

Conjugating bacterial cells obey the law (known as the surface exclusion) postulating that the recipient cell should not have the same conjugative plasmid as the donor cell. Otherwise, the frequency rate of conjugation will be very low, if it exists at all. Thus, the infertile homosexual conjugation (bacterial sodomy) between two donor cells is prohibited. The surface exclusion phenomenon depends on at least two genes located in the *tra* region of the F factor, *traT* and *traS*, which govern the synthesis of cell-wall and cytoplasmic proteins, respectively. These proteins stabilize mating pairs and presumably promote DNA transfer, unless the interacting cells are heterosexual. If this is the case, the TraT and TraS proteins hinder the formation of cell aggregates. The mutation of the *traT* and *traS* genes increases the frequency of formation of homosexual cell aggregates. One should not confuse the incompatibility of cells, which hinders the contact of two cells and the transfer of DNA into the female cell, and the incompatibility of two different plasmids that are already present in the same cell (not necessarily due to conjugation). In the latter case, one of the two plasmids is inevitably eliminated in daughter cells because of the replicative incompatibility of plasmids belonging to different incompatibility groups (the so-called *inc* groups).

A population of male cells always contains a small number of cells with a female phenotype, which carry the F factor but do not produce sex pili and thereby are unable to be DNA donors. This transient phenotype, whose frequency increases, for instance, in the late-logarithmic *E. coli* culture, is likely to be due to nonfunctional *tra* genes.

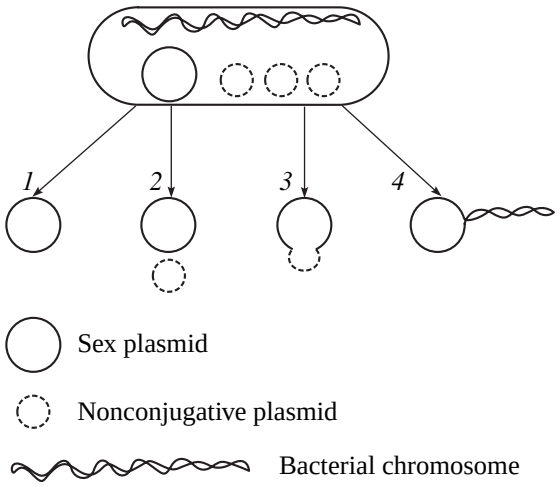
In any case, at the first step of conjugation, an open channel or pore is formed between two conjugating heterosexual cells, through which the male DNA (in the simplest case, the F factor) can pass into the female cell. This event, however, is preceded by the second step of conjugation, lying in the formation of a DNA–protein complex (the so-called relaxosome), which introduces a single-strand nick at the *nic* site of the F factor within the *oriT* recognition region and induces unwinding of the supercoiled DNA at this site. The relaxosome contains the enzyme relaxase (with the properties of helicase) encoded by the *traI* gene of the F factor, as well as some proteins with endonucleolytic activity. At the third step of conjugation, the nicked DNA strand of the F factor is transferred into the recipient cell with ATP as the source of energy.

At the third conjugation step, an important role is played by the TraD protein molecules, localized between the cell wall and the cytoplasmic membrane at the site of pore formation. TraD proteins, which belong to a family of TraG-like proteins, are formed by various conjugative plasmids. The interaction of the TraD proteins with the relaxosome has recently been reported by Szpirer *et al.* [12]. The transfer of the single-strand DNA into the donor cell is accompanied by the strand displacement DNA synthesis in the donor cell and the synthesis of a complementary DNA strand in the recipient cell. After the completion of DNA synthesis, the female cell acquires a copy of the F factor, thereby changing its sex to male. On the other hand, the donor cell retains its F factor due to replacement synthesis and remains male as well.

The F factor is not only capable of self-transmission but is also able to promote the transfer of other replicons, such as nonnconjugative plasmids and chromosomes. Nonnconjugative plasmids can be transferred either by conduction or by donation. In the case of conduction, the F factor forms a composite structure (or cointegrate) with a nonnconjugative plasmid (such as pBR322) with the involvement of the *Tn*1000 transposon located on the F factor. Then the cointegrate is transferred into a recipient cell, where it breaks down into components. Donation is the transfer of a nonnconjugative plasmid when a cointegrate is not formed and can occur even if the F factor and nonnconjugative plasmids (such as ColE1) do not have sequences (transposons or sufficiently extended homologous regions) necessary for the formation of cointegrates. Of interest is the fact that some mutations of the *tra* genes inhibit the transfer of the F factor itself but do not block the transfer of plasmid ColE1 by the mechanism of donation. The first conjugation step (i.e., the formation of sex pili and the pilus-mediated contact of mating cells) is necessary for donation to occur. This step is implemented by the conjugative plasmid. The second step of conjugation (the formation of relaxosome and DNA nicking and unwinding) can occur independently of the F factor, since many small plasmids have the *oriT* region and are able to produce Mob proteins, which are homologues of relaxase. The third step of conjugation by donation (the transfer of donor DNA into the recipient cells) again requires conjugative plasmids, since the necessary coupling proteins are produced, as a rule, only by such plasmids.

The F factor integrated into the host cell chromosome with the aid of transposable elements or by means of homologous recombination promotes the transfer of

this chromosome into a female cell (formally, such a transfer is a particular case of conduction). The transfer of the *E. coli* chromosome, which is 35 times longer than the sex plasmid, lasts about 100 min and can be interrupted at any time (most frequently, the chromosome fails to be completely transferred). Figuratively, the F factor works as a locomotive pushing ahead a long train and entering, if ever, the female cell after the last wagon. The transfer of chromosome with the aid of the F factor was studied in a great number of works, whose consideration is beyond the scope of this review, especially as there is no evidence that bacillar chromosomes can also be transferred through conjugation. The different mechanisms of F-factor transfer into female cells are depicted in the figure.



Different types of the conjugal transfer of replicons with the involvement of the F factor: (*1*) the transfer of the sex plasmid (the F factor) alone, (*2*) the transfer of the sex and nonconjugative plasmids without forming a cointegrate (donation), (*3*) the transfer of the sex and nonconjugative plasmids with the formation of a cointegrate (conduction), and (*4*) the transfer of the sex plasmid and bacterial chromosome with the formation of a cointegrate.

Bacterial conjugation systems are very diverse. In addition to the F factor and its variants and derivatives, *E. coli* can implement conjugation with the aid of other conjugative plasmids, such as R factors and Col plasmids. Conjugation in different bacteria may differ not only in the length and morphology of sex pili. For instance, the same salmonella cell may have either short (for mating on a solid surface) or long pili (for mating in liquid media). Bacilli and some other bacteria can come into mating contact without the involvement of pili (see below). Some mating bacteria, such as enterococci, can form very large aggregates, which comprise thousands of cells and are easily seen by the naked eye [13, 14]. It should be noted that a peculiarity of enterococci is that they have a complex pheromone system, which determines which cells will be DNA donors and which will be DNA recipients [15].

Conjugative transposons, another type of sex plasmids, have recently been reviewed by Kameneva [16]. These transposable elements emerge from bacterial chromosomes, forming circular plasmidlike structures. Transposons have a size (60–100 kb) similar to that of many conjugative plasmids and contain the extended *tra* region, the *mob* gene, and the *oriT* region. The Mob protein produced by transposons is able to bind to *oriT* and to induce single-strand nicks and relaxation. Like conjugative plasmids, conjugative transposons are capable of self-transmission and the transfer of small nonnconjugative plasmids by the mechanisms of donation and conduction.

Conjugation is possible between bacterial cells belonging to different species and genera, between bacterial and plant cells (an example is the transfer of the Ti plasmid from agrobacteria to plant cells), between bacteria and yeasts (e.g., between *E. coli* and *Saccharomyces cerevisiae* [17]), and even between bacterial and mammalian (HeLa) cells [18].

Before turning to the problem of conjugation in bacilli, let us briefly consider some terminological problems. Sometimes bacterial and, especially, bacillar conjugation is called transconjugation or transception, while a recipient cell with an acquired plasmid is called transconjugant or transcipient. I do not adhere to this terminology and instead will use the terms mobilization (to designate the transfer of nonnconjugative plasmids, particularly due to donation) and mobilizable plasmids (to designate plasmids that became transposable in the course of conjugation).

2. CONJUGATION IN THE *B. CEREUS* AND *B. SUBTILIS* GROUPS

Although bacilli comprise a great number of genera and species, conjugation was studied in representatives of only two bacillar groups, the group of *B. cereus* and the group of *B. subtilis.*

2.1. The B. cereus Group

This group includes three species, *B. cereus, B. thuringiensis,* and *B. anthracis.* The G + C content in the DNA of these bacilli is 34–35 mol %. *B. thuringiensis* is the most industrially important bacterium of this group, as it is a producer of delta-endotoxin and thermostable beta-exotoxin, which possess insecticidal activity. These toxins kill caterpillars and the larvae of blood-sucking dipteras and beetles by damaging their intestines. *B. thuringiensis* spore preparations are sprayed as biopesticides at pest-contaminated sites. Delta-toxin is synthesized in the phase of spore formation and may comprise up to 30% of the total cellular protein. Due to its high concentration in cells, this toxin occurs in the form of large crystals, occupying about one-third of the total cell volume. The genes that control toxin synthesis are mainly localized on large plasmids. Some strains of *B. thuringiensis* have up to

17 plasmids from 1–2 to 300–400 kb in size, which comprise 20–25% of the total cellular DNA. The complex plasmid profile of *B. thuringiensis* makes it very difficult to determine the function of the particular plasmids of this bacterium. Some strains of *B. thuringiensis* do not produce toxins [19, 20]. There are more than 20 subspecies of *B. thuringiensis*, which differ in the serotypes of flagellar antigens and in some other characteristics.

B. anthracis is important in human and veterinary medicine, as it is the causal agent of anthrax. Like the *B. thuringiensis* toxins, the *B. anthracis* toxins are mainly encoded by plasmid genes.

B. cereus can grow in foodstuffs and may cause bacterial food poisoning.

The conjugal transfer of plasmids in *B. thuringiensis* was discovered in the laboratory headed by B. Carlton at the University of Georgia, the United States [21, 22]. The transfer of large plasmids (60–80 kb) was observed when donor cells bearing these plasmids were grown in a liquid medium together with recipient cells bearing other plasmids, including a streptomycin-resistance plasmid (this allowed the colonies grown from the donor and recipient cells to be distinguished). The presence of foreign plasmids in the recipient cells was detected by electrophoresis and by the immunologic reaction of plasmid-encoded delta-endotoxin with its antibodies. The frequency of the conjugal transfer of large plasmids between the donor and recipient cells was very high (up to 75%). Researchers from this laboratory also detected the transfer of small cryptic plasmids to the recipient *B. thuringiensis* cells and the large plasmids of *B. thuringiensis* to the recipient *B. cereus* cells.

Investigations along these lines were also carried out in the laboratory headed by C. Thorne at the University of Massachusetts, the United States [23, 24]. The donor strains *B. thuringiensis* subsp. *thuringiensis*, *morrisoni*, *toumanoffi*, *alesti*, and *israelensis* carried large plasmids pXO11 through pXO16 from 80 to 190 kb in size (some of which encoded toxin synthesis) and the small tetracycline resistance plasmid pBC16, which was isolated from *B. cereus* and introduced into the donor strains by means of transduction. The recipient strains of *B. thuringiensis*, *B. cereus*, and *B. anthracis* were resistant to streptomycin. To detect the mobilization of plasmid pBC16 during conjugation in a liquid medium, colonies resistant to both streptomycin and tetracycline were selected. Depending on the recipient, the frequency of pBC16 transfer varied from 10^{-1} to 10^{-8} per recipient cell, but it was in most cases high. The recipient cells with the transferred plasmid pBC16 were found to contain also large plasmids and small cryptic plasmids from the donor cells. Conjugants with the large plasmids acquired the properties of donor cells. When in *B. anthracis* cells, the large plasmid pOX12 promoted the conjugal transfer of some large plasmids of *B. anthracis* controlling the synthesis of the anthrax toxin and the virulence factor of this bacterium.

To elucidate which of the numerous plasmids of *B. thuringiensis* are responsible for toxin synthesis, researchers from the laboratory headed by R. Azizbekyan at the Institute of Genetics and Selection of Industrial Microorganisms, Moscow, Russia, carried out experiments on the conjugal transfer of large plasmids from the toxin-producing donor strains of *B. thuringiensis* into the recipient *B. cereus* strain. The donor strains were marked by introducing the erythromycin resistance plasmid pAMβ1. The recipient strain was resistant to tetracycline due to spontaneous mutation. Conjugation was carried out both in a liquid medium and on the membrane surface. Conjugants resistant to both antibiotics were examined by electrophoresis for the presence of large plasmids. It was found that the conjugants, which were produced at a low frequency rate (10^{-5} to -10^{-6}), also carried large plasmids and produced toxin. Enzyme immunoassay showed that the toxin produced by the conjugant *B. cereus* cells was identical to that produced by the donor *B. thuringiensis* cells. It was likely that plasmid pAMβ1 promoted the mobilization of some plasmids of *B. thuringiensis*. An electron microscopic analysis of the conjugation mixture showed the presence of short bridges and fused regions between the surface layers of cells, although convincing evidence that these cells are conjugants was not presented [25, 26]. It should be noted that the transfer of the bacteriocin-producing ability between different *B. thuringiensis* strains during mixed cultivation was first described as far back as 1979 (without discussing the mechanism of this transfer) [27]. The important role of plasmid pAMβ1 in the transfer of toxin-encoding plasmids was also shown by Lereclus *et al.* [28].

The most comprehensive investigations of conjugation in *B. thuringiensis* were carried out in the laboratory headed by L. Andrup at the National Institute of Occupational Health, Copenhagen, Denmark. *B. thuringiensis* was studied as a potential contaminant of the respiratory and gastrointestinal tracts of greenhouse workers dealing with pesticide preparations containing spores of this bacterium. The bacterium found in the organisms of the workers proved to be harmless, but its investigation was continued with reference to conjugation and some other aspects [29–37].

Experiments were carried out with the *B. thuringiensis* subsp. *israelensis* and *kurstaki*. The donor strain carried a large conjugative plasmid, pXO16 (200 kb), and, in some experiments, conjugative plasmids pHT73 and pAW63. To facilitate the detection of conjugative plasmids, they were marked by the chloramphenicol resistance transposon *Tn*5401. Plasmid pBC16 and some small plasmids constructed on the basis of vectors with the sigma- and theta-type replication served as mobilizable plasmids. Conjugation was observed in a liquid medium and required the presence of plasmid

pXO16 in one of the mating partners. Cells that lost this plasmid, for instance, as a result of heating to a temperature of more than 40°C, were incapable of conjugation. On the other hand, conjugation was not observed when both donor and recipient cells contained the same conjugative plasmid (i.e., the surface exclusion mechanism was functional). Plasmid pXO16 was transferred at a very high frequency, so that almost all recipient cells received it. Plasmid pBC16 was transferred at a much lower frequency rate (10^{-3} to 5×10^{-3} per recipient cell). Conjugating bacteria formed large aggregates, which contained thousands of cells and were visible to the naked eye. Electron microscopic analysis showed the presence of very short bridges between two or sometimes more mating cells [32]. Aggregation was observed only for cells of which one contained plasmid pXO16 and the other lacked it. In monocultures of recipient and donor cells, intercellular bridges were not formed. Thus, cell aggregation proved to be the first step of conjugation. According to the data of electron microscopy, the structure of the cell walls at the sites of cell-to-cell contact was altered (personal communication of G. Jensen).

The treatment of plasmid-free cells with proteinase K prevented their aggregation with plasmid-bearing cells. Presumably, conjugative plasmid imparted the Agr^+ phenotype to bacterial cells, while female cells had the Agr^- phenotype.

The transfer of conjugative plasmids began as soon as donor and recipient cells came into contact and lasted for 3.5–4 min. After each coupling event, the donor cell required a 10-min relaxation period to be capable of the next coupling act, while the recipient cell required a 40-min period to become able to donate the acquired plasmid (i.e., to become a male cell). Conjugation bridges could be formed between one donor cell and several recipient cells; however, large plasmids were transferred only to one of the recipient cells. The transfer of two small nonnconjugative plasmids, pC194 and pE194, to one recipient cell at a time was a rare event (7×10^{-7} per recipient cell). Conjugation was most efficient when the proportion between the donor and recipient cells was 1 : 200. The relationship between the efficiency of conjugation and the concentration of recipient cells could be described by the Michaelis–Menten equation [37]. The phenomenon of cell competence, such as that observed in the growing *B. subtilis* culture, was not revealed. Nor were pheromones that stimulate conjugation detected.

In addition to the native plasmid pBC16, researchers also used its derivatives with deletions and inversions in the *mob* gene and the *oriT* region, plasmid pX14-3 with the sigma-type replication and its derivatives with an altered structure of the *mob* gene, and the truncated pLS20 and pAMβ1 plasmid derivatives with theta-type replication. Experiments showed that alterations in the *mob* gene reduced the mobilization frequency no more than tenfold (it should be noted that the reduction of the mobilization frequency could be due not only to the gene alteration itself but also to a smaller number of pLS20 and pAMβ1 copies, their slow replication, and other reasons). With the other conjugative plasmid studied, pAW63, the mobilization of plasmid pBC16 required the native *mob* gene. The presence of the *oriT* region was a necessary condition of mobilization. Plasmids with theta-type replication could be transferred from the donor cells that carried plasmid pXO16 [32].

The *B. thuringiensis* subsp. *thuringiensis* was able to transfer plasmid pXO16 to 14 different recipient *B. thuringiensis* subspecies and to *B. cereus*. Another conjugative plasmid, pAW63, could be transferred from *B. thuringiensis* subsp. *kurstaki* to the species *B. cereus*, *B. licheniformis*, *B. sphaericus*, and *B. subtilis* [34]. *B. thuringiensis* was also able to transfer plasmid pBC16 to a wide range of recipient bacillar species (*B. cereus*, *B. megaterium*, *B. brevis*, *B. subtilis*. *B. polymyxa*, and *B. sphaericus*) [38].

2.2. The B. subtilis Group

The G + C content of the DNA of this group averages 42.9%. The best studied representative of this group is the laboratory strain *B. subtilis* Marburg (or *B. subtilis* 168). The group also includes numerous soil isolates of *B. subtilis* and three subspecies, *B. subtilis* W23, *B. subtilis* (niger), and *B. subtilis* (natto), which are sometimes considered to be separate species. The species *B. pumilus*, *B. licheniformis*, *B. atropheus*, *B. mojavensis*, and *B. polymyxa* are also members of this group, since their DNA can transform the *B. subtilis* 168 cells, albeit with a much lower efficiency than the homologous DNA. About 30% of *B. subtilis* strains (but not *B. subtilis* 168) carry small cryptic plasmids about 10 kb in size (sometimes one strain can carry even two different plasmids) [39]. These small plasmids with sigma-type replication are very similar in their gene composition, although their host strains were isolated in different geographical areas [40]. Large cryptic plasmids (40 to 100 kb in size) with predominant theta-type replication are not so frequent (they are present in 5–7% of *B. subtilis* isolates) as small cryptic plasmids [40]. In general, the number of plasmids in representatives of the *B. subtilis* group is much smaller (and their role is not so important) than in *B. thuringiensis*.

B. subtilis 168 is widely used for laboratory and biotechnological purposes. *B. subtilis* (*natto*) is a starter strain in the production of natto (fermented soybeans very popular in Japan).

The studies of the conjugal transfer of plasmids in *B. subtilis* began in the early to mid-1980s. Klier *et al.* [41] described a hybrid plasmid constructed from the *B. thuringiensis* plasmid involved in toxinogenesis and the bifunctional *E. coli–B. subtilis* vector pHV33. The hybrid plasmid was introduced (by transformation) into *B. subtilis* 168, which was then incubated in a liquid

medium with an atoxic strain of *B. thuringiensis.* The toxin-producing conjugants were formed at a frequency rate of 10^{-4} to 10^{-5}. Oultram and Young investigated the conjugal transfer of the streptomycin-resistance plasmid pAMβ1 (26.5 kb) of *Streptococcus faecalis* and found that this plasmid was transferred, at a rate of 10^{-6} to 10^{-8}, from *S. faecalis* to *B. subtilis* 168 and from *B. subtilis* to *Clostridium acetobutylicum* and vice versa [42].

Koehler and Thorne investigated *B. subtilis* (natto) with the large plasmid pLS20 (55 kb), which was marked by lincomycin and streptogramin resistance by introducing transposon *Tn*917 [43]. The donor strain was marked by tetracycline resistance by introducing the tetracycline-resistance plasmid pBC16 into the regenerating protoplasts of this strain. This allowed the transfer of large conjugative and small nonnconjugative plasmids to be easily detected. Conjugation was performed on the membrane surface. When the donor cells contained plasmid pLS20, plasmid pBC16 was transferred to the pLS20-lacking recipient cells of the same strain at a frequency of 10^{-4} per donor cell. About 44% of the conjugants acquired plasmid pLS20 as well. In this conjugation system, also mobilizable were the small 5.4-kb cryptic plasmid pLS19 native to the donor strain and the small foreign plasmid pUB110 preliminarily introduced into the donor cells, while the small plasmids pC194 and pE194 were not mobilizable.

Plasmid pBC16 could also be transferred from *B. subtilis* (natto) to other bacillar species (*B. subtilis* 168, *B. licheniformis, B. thuringiensis, B. cereus*, and *B. megaterium*) at a frequency of 10^{-4} to 10^{-6}, depending on the species. The lowest transfer frequency was observed for *B. megaterium.* The large conjugative plasmid pLS20 could be transferred only to a *B. subtilis* strain deficient in restriction enzymes. Plasmid pBC16 was transferred by the mechanism of donation rather than by the mechanism of conduction. Indeed, if conduction (which is associated with the formation of the pLS20 and pBC16 cointegrate and its subsequent breakdown in the recipient cells) were the case, the structure of the plasmid pBC16 isolated from the recipient cells would have been altered. The failure of the transfer of the large plasmid pLS20 during interspecies mating can be accounted for by its restriction.

The mechanism of the conjugal transfer of small plasmids pBC16 and pUB110 mobilized by the large plasmid pXO503 was studied by Selinger *et al.* [44]. Plasmid pXO503 is a derivative of pLS20 with the inserted transposon *Tn*917 (see above). The donor strain contained plasmid pXO503 and the derivatives of the small plasmids pBC16 and pUB110. The recipient strain was a rifampicin-resistant *B. pumilus* strain. Mating was carried out on the membrane surface. The small native plasmids were mobilized at a rate of 2.5×10^{-4}, i.e., tenfold more frequently than the conjugative plasmid itself. To elucidate the necessity of the *mob* gene homologue (the so-called ORFβ) and the *oriT* site localized in the flanking region of this gene, fragments of some *E. coli* plasmids were inserted into the ORFβ region. The resultant hybrid plasmids were transferred, if at all, at least 100–200 times less frequently than the native plasmids. Deletions in the respective regions of pUB110 and pBC16 produced the same effect. Alterations in the other regions of the small plasmids did not influence their mobilization.

The detrimental effect of deletions and insertions in the *mob* region on the process of mobilization was greatly mitigated if the donor cells contained not only altered but also native plasmids, for instance, pUB110 together with pUCBB (a hybrid of pUB110 and pUC18). In this case, native plasmid and hybrid plasmid with the defective *mob* gene were mobilized at nearly equal frequency rates, indicating that the deficiency of the defective *mob* gene product was compensated for by the synthesis of this product from the plasmid with the native *mob* gene (in other words, the native *mob* gene was trans-active, likely due to the ability of its product (relaxase) to freely diffuse throughout the cell cytoplasm). But when the *oriT* region was defective, no complementation was observed, presumably due to the fact that, in order to be able to mobilize small plasmids, the product of the *mob* gene (irrespective of whether it is foreign or not) must interact with the *oriT* region of the transferred plasmid. The recombinational association of native and defective plasmids was not necessary, as is evident from the occurrence of mobilization in the presence of the *recE4* (*recA4*) mutation, which prohibits homologous recombination in *B. subtilis.*

Our experiments on conjugation in *B. subtilis* and other bacilli were carried out with soil isolates of *B. subtilis* bearing large plasmids. Donor strains were prepared by introducing the kanamycin-resistance plasmid pUB110 into the protoplasts of these isolates. The recipient strain was either the restriction-deficient *B. subtilis* strain RM125 or one of the *B. subtilis* 168 (BD170 trpC2 thr5) auxotrophs. The recipient strains had the chromosomal marker of chloramphenicol resistance, which allowed conjugants to be easily selected through their resistance to both chloramphenicol and kanamycin.

We succeeded in isolating at least two strains with large plasmids, *B. subtilis* 1387(p1387-3) and *B. subtilis* 19(p19) (the latter strain was isolated by M.A. Titok (Belarusian State University) from a forest soil in Belarus). The size of these plasmids was 36 and 95 kb, respectively. The frequency rate of the conjugal transfer of pUB110 with the aid of plasmid p1387-3 was very low (10^{-7}), the transfer being observed only to the restriction-deficient strain and only on solid media [45]. At the same time, the frequency rate of the conjugal transfer of pUB110 with the aid of plasmid p19 was quite high (10^{-2} per recipient *B. subtilis* cell). The mobilization of pUB110 did not require a deficiency of restriction enzymes in the recipient strain and occurred

at nearly equal frequency rates on solid and in liquid media [46–48].

To determine the frequency rate of the simultaneous transfer of plasmid pUB110 and the conjugal plasmid p19, we used kanamycin- and chloramphenicol-resistant conjugants as the donor and erythromycin-resistant *B. subtilis* cells as the recipient. None of the 100 conjugant colonies tested was able to transfer plasmid pUB110. This indicated that plasmid p19 was absent in the conjugants. Nor was it detected by electrophoresis. Plasmid p19 marked by the erythromycin-resistance transposon *Tn*917 could be transferred to the recipient *B. subtilis* 168 strain at a frequency rate of 10^{-7}. Such a low frequency rate explains why the transfer of plasmid p19 was not detected in experiments without the selection of conjugants (unpublished data). Thus, the mobilization of plasmid pUB110 accompanied by the transfer of p19 occurs at a very low frequency. It should be noted that cells containing both plasmids, p19 and pUB110, and grown in the presence of high kanamycin concentrations were prone to the elimination of p19 and were unable to mobilize pUB110 [49]. The reason for this is unknown.

In the next experiment, we used the plasmid p19–free strain *B. subtilis* 19 as the recipient and found that the mobilization of pUB110 was accompanied by the transfer of the large conjugative plasmid p19. The transfer of p19 in this experiment can be explained by the fact that the recipient and the donor belonged to the same *B. subtilis* strain 19 and, hence, had the same restriction–modification systems, which provided for better integrity of the large plasmid (p19) during its transfer from the donor to the recipient [50].

When the donor and the recipient contained the large plasmid p19, the mobilization frequency of plasmid pUB110 decreased tenfold [50], which was presumably due to the surface exclusion phenomenon (see above).

Experiments with the termination of plasmid transfer by vigorous shaking of the liquid conjugation medium showed that the transfer of plasmid pUB110 began not later than 20 min after the mating cells came into contact. The optimal temperature for plasmid mobilization was 30–37°C. At 21°C, mobilization occurred with a delay and was less efficient. Large cell aggregates visible to the naked eye were not formed. The treatment of cells with proteinase K diminished the efficiency of plasmid transfer no more than two- to threefold. Cells from the midexponential growth phase were most competent to plasmid transfer.

In the next experiment, the tetracycline-resistant plasmid pBC16 was introduced into the regenerating protoplasts of *B. subtilis* 19(p19). The native plasmid pBC16 was transferred to recipient cells with about the same frequency as plasmid pUB110. The pBC16 derivative with the inverted *mob* gene (the derivative was obtained from Jensen) was transferred four orders less efficiently, which was in agreement with the data of Andrup *et al.* [32] concerning plasmid transfer in *B. thuringiensis*. The presence of the *mob*-bearing small cryptic plasmid pV in the recipient cells augmented the transfer frequency of the pBC16 derivative by 100 times (presumably, due to the transaction of the "foreign" *mob* gene).

The ability of *B. subtilis* to mobilize plasmids with theta-type replication was studied using the truncated erythromycin-resistant derivative plasmid pCB20 (8 kb) of the large plasmid pSM19035 with the theta-type replication. It was found that this derivative plasmid was transferred four to five orders of magnitude less efficiently than plasmid pUB110 [50].

Experiments were carried out not only with *B. subtilis* but also with other bacillar species and gram-positive bacteria, such as *B. subtilis* (niger), *B. subtilis* (natto), *B. sphaericus*, *B. megaterium*, *B. polymyxa*, *B. licheniformis*, *B. globigii*, *B. thuringiensis*, *B. mesentericus*, *B. amyloliquefaciens*, *B. pumilus*, and *St. aureus*. All recipient strains were streptomycin-resistant. Conjugants were selected on media with kanamycin and streptomycin. It was found that plasmid pUB110 was transferred to all the bacillar strains studied except for *B. mesentericus*, the transfer to *B. subtilis* (natto) and *B. polymyxa* being even more efficient than to *B. subtilis* 168. The plasmid transfer to *B. sphaericus*, *B. globigii*, and *B. thuringiensis* was two to three orders of magnitude less efficient than to the other recipients studied. Experiments with staphylococci showed that plasmid pUB110 could not be transferred to these bacteria [50]. Sometimes, the incubation of *B. subtilis* together with other bacillar species inhibited the growth of one of the partners (presumably, because of the action of bacteriocins), which could influence the calculation of the mobilization frequency of pUB110. Similar effects accompanying heterologous bacterial mating were observed by other researchers.

2.3. The Conjugal Transfer of Plasmids in the Natural Habitats of Bacilli

It is likely that the conjugal transfer of large and small plasmids is one of the major mechanisms of their distribution in the natural habitats of bacilli. Direct evidence for the existence of such transfer was obtained for *B. subtilis* and *B. thuringiensis*. Thus, plasmid pBC16 was mobilized during the combined infection of some caterpillars with different *B. thuringiensis* strains [51, 52]. The transfer of the large conjugative plasmid pXO16 marked by the *Tn*504 transposon was observed between different *B. thuringiensis* strains incubated in riverine water or during the infection of mosquito larvae. Under these conditions, plasmid pBC16 was not mobilized [53]. The transfer of a conjugative plasmid was also observed between the spore-forming and non-spore-forming *B. thuringiensis* strains incubated in microcosms with sterile soil [54, 55]. The small plasmid pFT10 was transferred, albeit at a low rate, between *B. cereus* and *B. subtilis* in sterile soil and in

some types of nonsterile soil [56]. The conjugal transfer of plasmid pUB110 was observed (both at 30°C and room temperature) in microcosms with either sterile or nonsterile garden soil that was preliminarily contaminated with the vegetative cells or spores of *B. subtilis* 19 (p19 pUB110) [57]. It should be noted that nearly all small cryptic plasmids found in the soil isolates of *B. subtilis* contained the *mob* gene, suggesting that the conjugal transfer of bacillar plasmids is widely spread in nature [40, 58, 59]. There is evidence that conjugation is less sensitive to unfavorable environmental conditions than the other mechanisms of horizontal gene transfer in bacteria [1–3].

CONCLUSION

Although the literature dealing with conjugation in bacilli is limited (the list of relevant publications cited in this review comprises 40 references), it still allows some generalizations to be made. Conjugative plasmids in bacilli (especially in *B. thuringiensis)* are quite numerous. Their size varies from 40 to 150 kb. The frequency rate of conjugation varies widely. In many cases, conjugation occurs only on solid surfaces (membranes and agar media). Bacillar cells lack structures resembling the sex pili of gram-negative bacteria (at least, such structures have not yet been detected). When in contact, heterosexual bacillar cells form aggregates, which may comprise thousands of cells and, if so, can be seen by the naked eye. Cell aggregates are most likely formed with the involvement of proteins and other constituents of the cell surface, whose synthesis is regulated, directly or indirectly, by conjugative plasmids. The formation of pores at the site of the bacillar cell contact is not confirmed directly. The phenomenon of surface exclusion is manifested to a certain degree. The conjugal transfer of chromosomal genes has not yet been shown. At the same time, there is convincing evidence that large self-transmissible conjugative plasmids and small mobilizable plasmids can be transferred, albeit at different frequency rates and sometimes not simultaneously. The structure of bacillar conjugative plasmids and the mechanism of their transfer have been far from adequately studied, which is a serious gap in the research on bacillar conjugation. Some large conjugative plasmids, such as pMRC01 of *Lactobacillus lactis* [60] and pSK41 of *Staphylococcus aureus* [61] have been completely sequenced. It was found that the *tra* region of the 60-kb plasmid pMRC01 has 16 genes, whereas the 15-kb *tra* region of the 46.4-kb plasmid pSK41 has 13 genes. Similar sizes of conjugative plasmids and their *tra* regions can be anticipated for bacilli. It should be noted that fragments of some replicons of large bacillar plasmids have already been sequenced [62, 63].

The mobilization of small bacillar plasmids likely does not require the formation of recombinational cointegrates with conjugative plasmids and, hence, does not occur through the mechanism of conduction. Consequently, in bacilli, as in other gram-positive bacteria, donation is the major mechanism of plasmid transfer. In any case, the presence of the *mob* gene or its homologues is a necessary condition of plasmid mobilization in bacilli. The synthesis of relaxase (the *mob* gene product) can be controlled not only by the *mob* gene of the transferred plasmid but also by the homologous gene of another small plasmid. The origin of the signal that triggers the functioning of the *mob* gene of small plasmids is unknown, although it was suggested that such signal may come from large conjugative plasmids. Experiments performed in Andrup's and our laboratories showed that small plasmids can also be mobilized even if the *mob* genes of these plasmids are defective. It remains to be elucidated whether these defective genes performed their function at the expense of the function of other plasmids (including conjugative) or whether the migration of small plasmids to the recipient cells in this case was due to alternative mechanisms.

To conclude, the conjugal transfer of plasmids in bacilli is a rapidly developing line of research. Bacillar conjugation and the F factor–dependent conjugation in *E. coli* have much in common. For this reason, the investigation of bacillar conjugation is of general scientific significance. Manipulations on the conjugal transfer of plasmids in bacilli are rather simple and can easily be used for biotechnological purposes. Of great interest is the conjugal transfer of chromosomal genes in bacilli, although, to the best of our knowledge, there is only one publication devoted to this problem [64]. Some difficulties that researchers may face in dealing with this problem are related to the action of restriction enzymes, which prevent the transfer of large fragments of chromosomal DNA between different mating strains and species. This can be surmounted by searching for transposon-containing conjugative plasmids that will be able to form cointegrates of plasmids and chromosomes. Transposons and IS elements of such a type have recently been found by Nagai *et al.* [65] and by researchers from Bron's and our laboratories [66, 67].

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