Microbiology (1999), **145**, 2001–2009 Printed in Great Britain

A comparison of the kinetics of plasmid transfer in the conjugation systems encoded by the F plasmid from *Escherichia coli* and plasmid pCF10 from *Enterococcus faecalis*

Lars Andrup and Katja Andersen

Author for correspondence: Lars Andrup. Tel: +45 3916 5223. Fax: +45 3916 5201.
e-mail: andrup@internet.dk

National Institute of Occupational Health, Department of Microbiology, Lersø Parkallé 105, DK-2100 Copenhagen, Denmark

Quantitative measurements of horizontal DNA transfer are critical if one wishes to address questions relating to ecology, evolution and the safe use of recombinant bacteria. Traditionally, the efficiency of a conjugation system has been described by its transfer frequency. However, transfer frequencies can be determined in many ways and may be sensitive to physical, chemical and biological conditions. In this study the authors have used the mechanistic similarity between bacterial conjugation and simple enzyme catalysis in order to calculate the maximal conjugation rate (V_{max}) and the recipient concentration (K_m) at which the conjugation rate is half its maximal value, for two different conjugation systems: the F plasmid from *Escherichia coli* and plasmid pCF10 from *Enterococcus faecalis*. The results are compared with the data obtained from the aggregation-mediated conjugation system encoded on pXO16 from *Bacillus thuringiensis*. The conjugation systems analysed are fundamentally different; however, they have some characteristics in common: they are able to sustain conjugative transfer in liquid medium and the transfer efficiencies are very high. Conjugation encoded by the F plasmid in *E. coli* involves the formation of small aggregates (2–20 cells), established by sex pili, and the plasmid's maximal conjugation rate was estimated to be approximately 0·15 transconjugants per donor per minute. Pheromone-induced conjugation in *Ent. faecalis*, which involves the formation of large aggregates, was found to proceed at a maximal conjugation rate of 0·29 transconjugants per donor per minute. Also, the K_m value differed significantly between these conjugation systems; this may reflect the inherent differences in mating pair formation and transfer mechanisms. In these conjugation systems, the donors underwent a 'recovery period' between rounds of conjugative transfer and newly formed transconjugants required a period of about 40–80 min to mature into proficient donors.

Keywords: conjugation, pXO16, F plasmid, pCF10, pheromone

INTRODUCTION

Horizontal gene transfer is an important adaptive mechanism for bacteria that may result in increased genetic variation by bringing together DNA from different genetic backgrounds. Quantitative measurements of horizontal DNA transfer are critical if one wishes to address questions relating to the ecology and evolution of bacteria, such as the spread of antibiotic-resistance determinants or risks associated with the release of genetically engineered micro-organisms. The frequency and mechanisms of gene transfer between micro-organisms in nature are not well understood. Conjugation is probably the major mode of exchange of genetic information in the natural, physical environment (Yin & Stotzky, 1997) and in the intestinal tract of humans and animals (Schmidt *et al*., 1996; Jarrett & Stephenson, 1990; Salyers, 1993). Most known conjugation systems are encoded by large plasmids; however several conjugative transposons have been discovered (Salyers *et al*., 1995). The bulk of research has focused on Gram-negative conjugation systems and the

F plasmid in particular, but several conjugation systems from Gram-positive bacteria have recently attracted much attention (Clewell, 1993b). In contrast to conjugation systems in Gram-negative bacteria, those discovered in Gram-positive bacteria involve no pili, and other means of establishing and maintaining mating pair formation are employed. While the majority of conjugation systems in Gram-positive bacteria require solid surfaces to sustain mating pair formation, a few conjugation systems have been discovered in which aggregation between donor and recipient cells enables DNA transfer in liquid medium (Dunny *et al*., 1978; van der Lelie *et al*., 1991; Gasson *et al*., 1992; Andrup *et al*., 1993; Wirth, 1994).

The best-studied conjugation system in Gram-positive bacteria is pheromone-induced conjugation in *Enterococcus faecalis*. This elegant example of prokaryotic communication and flow of genetic information was discovered 20 years ago (Dunny *et al*., 1978), and has now become the paradigm for more than 30 pheromone-induced conjugation systems in *Ent. faecalis*. Pheromones, which are secreted by potential recipient cells, induce cell aggregation and transfer functions of donors carrying the relevant conjugative plasmid. This mechanism of mating pair formation and conjugative transfer is very efficient. Transfer frequencies of >1 (transconjugants per donor) have been reported in 2 h matings, using the tetracycline-resistance plasmid pCF10 (Dunny *et al*., 1982). Plasmid-free recipients secrete multiple sex pheromones, each triggering the relevant donor cell to express transfer functions and signalling that recipients are in close proximity. The pheromones produced by *Ent. faecalis* recipients are short hydrophobic peptides (7 or 8 amino acids) excreted in tiny amounts. Concentrations as low as 1–10 molecules per donor cell can induce aggregation of the donor cells. Recent reviews on pheromone-induced conjugative plasmids have been published by Clewell (1993a), Wirth (1994) and Zatyka & Thomas (1998).

We have previously reported the discovery and characterization of an aggregation-mediated conjugation system encoded on a large plasmid pXO16 (200 kb) in *Bacillus thuringiensis* subsp. *israelensis* (Andrup *et al*., 1993; Jensen *et al*., 1995). In addition to being able to mobilize any non-conjugative plasmid tested (Andrup *et al*., 1996) pXO16 transfers itself to practically every recipient cell in the mating mixture in a few hours broth mating and converts them to donor cells (Jensen *et al*., 1996). This non-pheromone-induced and protease-sensitive conjugation system in *B. thuringiensis* is a new and fundamentally different example of gene transfer involving communication between sexually differentiated cells.

In order to describe the spread of plasmids in quantitative terms, we have used a mathematical model based on the resemblance of bacterial conjugation to enzyme kinetics (Andrup *et al*., 1998). The process of conjugative plasmid transfer can be regarded as a catalytic conversion of recipients to transconjugants, mediated by the donors. We thereby obtain a simplified picture of the conjugation process, which closely resembles classical enzyme kinetics: donor and recipient are analogous to the enzyme and the substrate, respectively; the mating pair is analogous to the activated complex; and the transconjugant is analogous to the product. This model enables us to characterize a conjugation system by means of two constants: V_{max}, the maximal conjugation rate (the number of transconjugants produced per donor per minute); and K_m, which is the recipient concentration at which the conjugation rate is half its maximal value.

We have previously determined fundamental parameters for the conjugation system encoded on pXO16 in *B. thuringiensis* (Andrup *et al*., 1998). The observations can be summarized as follows: (i) The conjugative transfer takes about 3·5–4 min. (ii) The ability to transfer the plasmid seems to be evenly distributed among donors. (iii) Functionally, the mating complex consists of one donor and one recipient cell, even though aggregates comprising thousands of interconnected cells are formed. (iv) Having donated its plasmid, the donor requires a 'recovery period' of about 10 min before it can donate again. (v) Secondary transfer, i.e. transfer from newly formed transconjugants, only occurs about 40 min after transfer. Analysing the correlation of the conjugation rate to recipient concentration, using a model similar to Michaelis–Menten enzyme kinetics, showed that donor transfer activity has a saturation point. The biological significance of these findings is that at lower densities of recipients, their availability is transfer-limiting and the number of transconjugants is therefore proportional to the concentration of recipients, whereas at higher recipient concentrations, the intrinsic capacity of the conjugation system, V_{max}, becomes rate limiting.

The aim of the present work was to analyse two fundamentally different conjugation systems by the use of the kinetic model for conjugative transfer previously described (Andrup *et al*., 1998), and to compare the data with the results obtained for the aggregation-mediated conjugation system encoded on plasmid pXO16 from *B. thuringiensis* (Andrup *et al*., 1998).

METHODS

Strains and plasmids. For *E. coli* matings, strain MC1000 (F^-, Str^R) (Casadaban & Cohen, 1980) was used as recipient and strain XL-1 Blue (F^+::Tn*10*, Tet^R Nal^R) (Bullock *et al*., 1987) as donor. For *Ent. faecalis* matings, strain OG1-SSp (Str^R) (Dunny *et al*., 1982) was used as recipient and strain OG1-RF1 (pCF10, Rif^R Tet^R) (Dunny *et al*., 1978) as donor. Antibiotics were used at the following concentrations: streptomycin, 1000 μg ml^{-1} (*Ent. faecalis*) and 100 μg ml^{-1} (*E. coli*); tetracycline, 6 μg ml^{-1} (*Ent. faecalis*) and 10 μg ml^{-1}(*E. coli*); nalidixic acid, 15 μg ml^{-1}; rifampicin, 50 μg ml^{-1}.

Broth matings. Overnight cultures of the donor and recipient strains, grown separately at 37 °C in LB medium (*E. coli*) or BHI medium (*Ent. faecalis*) containing the appropriate antibiotics, were diluted 1:100 into fresh pre-warmed (37 °C) medium without antibiotics. *E. coli* matings were performed in 7 ml LB medium by combining 0·5 μl per OD_{600} unit of

donor cells in the exponential growth phase (OD_{600} between 0·4 and 0·6) and increasing numbers of recipient cells also in the exponential growth phase. The mating mixtures were incubated at 37 °C with moderate shaking (200 r.p.m.). At the time indicated, samples were taken, vortexed for 5 s, and dilutions (maximal volume 100 µl, at least three different plates) were plated on appropriate selective media and incubated overnight to determine the number of transconjugants. *Ent. faecalis* matings were conducted in 25 ml Erlenmeyer flasks with baffles containing a total volume of 15 ml. The *Ent. faecalis* donors (0·5 µl per OD_{600} unit) were induced by the addition of 3 ml pheromone medium (see below) and allowed an induction period of 60 min prior to the addition of recipients. The matings were incubated at 37 °C with moderate shaking (200 r.p.m.). Donors and recipients grown separately were included as controls. The numbers of donor and recipient cells in both kinds of matings (N_0) were determined at the start of the mating (t_0) and the number of cells (N_i) at a given time (t_i) was calculated using the relation $N_i = N_0 e^{kt_i}$, where $k = \ln(2)/(\text{generation time})$, assuming exponential growth.

Determination of conjugation rate. The rate of conjugative transfer was measured as the number of transconjugants produced per donor per minute over a period of 15 min in broth matings, allowing for an initial lag-phase and ensuring the production of a statistically significant number of transconjugants (e.g. after 30 and 45 min). The donor concentration used in this calculation was an estimate of the numbers at the midpoint of the two sample times (e.g. 37·5 min). The samples were taken before newly formed transconjugants matured into proficient donors.

Preparation of pheromone medium and determination of maximal induction. Medium containing recipient-produced pheromone was prepared essentially as described by Dunny *et al*. (1979). An overnight culture of the *Ent. faecalis* recipient strain (OG1-SSp), grown at 37 °C in BHI medium containing the appropriate antibiotic, was diluted 1:1000 into 400 ml fresh prewarmed (37 °C) BHI medium without antibiotics. At $OD_{660} \approx 1$ the supernatant was collected after centrifugation, sterile filtered (0·2 µm filter), autoclaved, and stored at 4 °C. To estimate the volume needed for maximal induction of the donors, various volumes (0, 0·05, 0·1, 0·2, 0·4, 0·8, 1·6 and 3 ml) of pheromone medium were combined with BHI medium in test tubes in a total volume of 7 ml. The test tubes were inoculated with 100 µl per OD_6 unit of the exponentially growing donor strain (OG1-RF1) and incubated for 60 min at 37 °C with shaking (200 r.p.m.). Then 300 µl per OD_{600} unit of the recipient strain, in exponential growth, was added to each tube and the mating mixtures were incubated for 45 min. Dilutions (maximal volume 100 µl) were plated on appropriate selective media for determining the number of transconjugants. Separately grown donor and recipient controls were included and used to determine the inoculum size and to verify that no streptomycin- and tetracycline-resistant colonies were formed in monocultures.

Mathematical model. The Michaelis–Menten model accounts for the kinetic properties of many enzymes and it states that the 'rate of catalysis', V, varies with the substrate concentration, [S], according to the following equation:

$$V = V_{\max} \frac{[S]}{[S] + K_m}$$

K_m is the Michaelis constant, which is equal to the substrate concentration at which the reaction rate is half its maximal value, V_{max}. The model was applied to the conjugation systems by considering the donor and the recipient as being analogous to the enzyme (E) and the substrate (S), respectively, the mating pair being analogous to the activated complex (E–S), and the transconjugant analogous to the product (P). The maximal conjugation rate, V_{max}, and K_m were calculated using a computer program (kindly provided by Bjørn Andersen Nexø, National Institute of Occupational Health, Denmark) based on the non-linear regression methods described by Wilkinson (1961).

RESULTS

E. coli matings

When analysing the kinetics of conjugative plasmid transfer it is important to differentiate between the contribution from the original donors in the mating mixture and the conjugative activity carried out by newly formed transconjugants. For F-plasmid-mediated conjugation in *E. coli*, it has been reported that most newly formed transconjugants are only able to mate after about 90 min (Cullum *et al*., 1978) and in the conjugation system encoded on the *B. thuringiensis* plasmid pXO16 a transconjugant maturation time of about 45 min has been established (Andrup *et al*., 1998). To verify that transconjugants formed in the early phase of a mating arise from transfer activity carried out by the

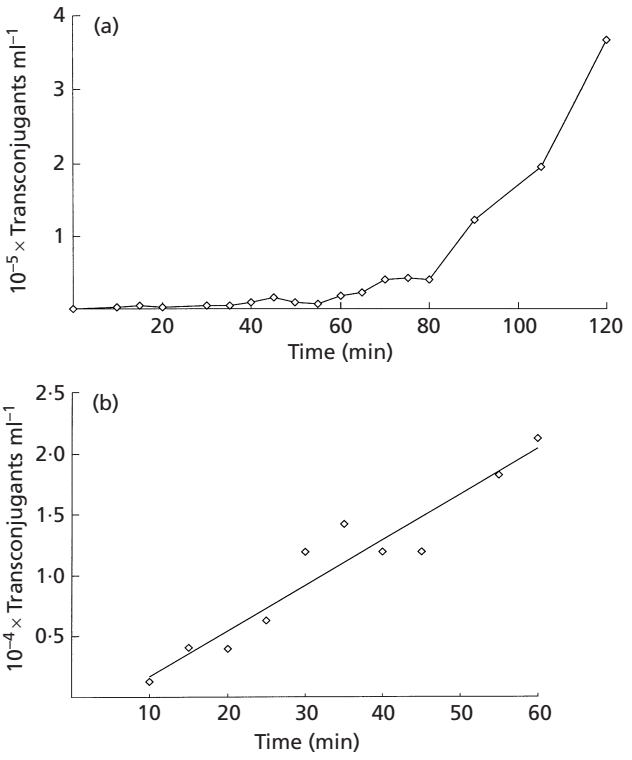

Fig. 1. Appearance of transconjugants in F-plasmid-mediated conjugation in *E. coli* as a function of time. (a) Donors ($4·1 \times 10^3$ cells ml^{-1}) were combined with an excess of recipients ($7·6 \times 10^6$ cells ml^{-1}). (b) Donors ($1·5 \times 10^3$ cells ml^{-1}) were combined with an excess of recipients ($5·1 \times 10^6$ cells ml^{-1}), focusing on the first 60 min of the mating. The linear regression of the data is shown ($R^2 = 0·92$; $\alpha = 374$). Transconjugants were selected on agar plates containing streptomycin and tetracycline.

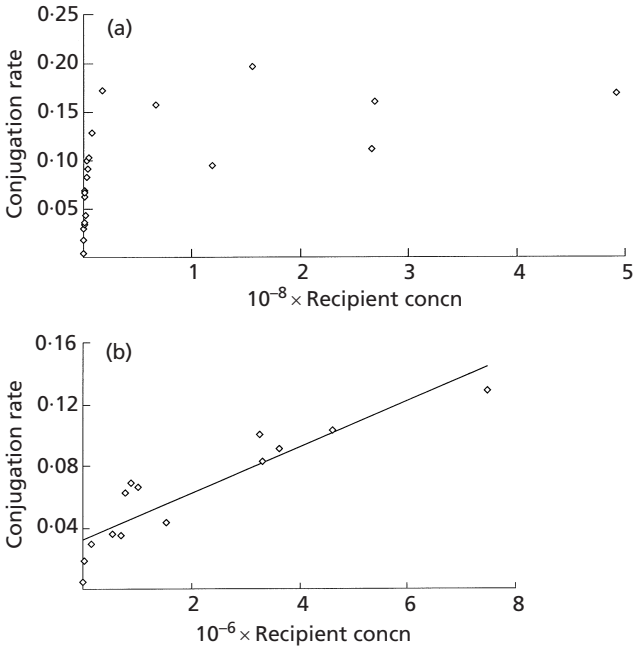

Fig. 2. Correlation between conjugation rate (transconjugants per donor per minute) and recipient concentration (c.f.u. ml^{-1}) in F-plasmid-mediated conjugation in *E. coli*. The donor concentration was $5{\cdot}1 \times 10^3 \pm 3{\cdot}2 \times 10^3$ at the start of the matings. The recipient concentration was calculated using the method described in Methods. (b) The 14 mating experiments at the lowest recipient concentration presented in (a) showing the proportionality between conjugation rate and recipient concentration ($R^2 = 0{\cdot}82$).

original donors, the kinetics of transconjugant formation were determined in mating experiments with a large excess of recipients. In Fig. 1, the number of transconjugants in the mating mixture is represented as a function of time and it can be seen that the appearance of transconjugants was more or less linear up to about 70–80 min after the onset of mating. At about 80 min, the formation of transconjugants increased dramatically due to the conjugation activity carried out by the now donor-proficient transconjugants. The data shown in Fig. 1(b) emphasize the proportionality ($R^2 = 0{\cdot}92$) of the appearance of transconjugants in the first 60 min of a mating period. Using the estimated generation time of the donors of $42{\cdot}5 \pm 3{\cdot}2$ min, the numbers of donors ml^{-1} could be calculated as approximately $2{\cdot}6 \times 10^3$ at $t = 35$ min. The conjugation rate was calculated, using the slope of the line in Fig. 1(b), as 0·14 transconjugants per donor per minute.

Correlation between recipient concentration and conjugation rate

To determine the correlation between conjugation rate and recipient concentration, a series of 21 mating experiments with recipient concentrations in the range 1×10^3 to 5×10^8 cells ml^{-1} was conducted. The donor concentration was held relatively constant ($5{\cdot}1 \times 10^3 \pm 3{\cdot}2 \times 10^3$). The estimated conjugation rates, determined as the number of transconjugants produced per donor per minute, are plotted in Fig. 2. As seen in Fig. 2(a), an upper limit in conjugation activity was reached when recipient concentrations exceeded approximately 2×10^7 cells ml^{-1}. The correlation between conjugation rate and recipient concentration was more or less linear in the lower recipient concentration range ($R^2 = 0{\cdot}82$) (Fig. 2b). Using the mathematical model based on the resemblance between the kinetics of bacterial conjugation and enzyme reaction kinetics (Andrup *et al.*, 1998) the maximal conjugation rate (V_{max}) and the recipient concentration at which the conjugation rate was half its maximal value (K_m) were calculated. The results are presented in Table 1.

Table 1. Comparison of the three conjugation systems

Conjugative plasmid (original host)	Size (kb)	Size of aggregates*	Auto-aggregation†	Induction by pheromones	Pili	V_{max}‡	K_m§
F (*E. coli*)	100	2–20	no	no	yes	$0{\cdot}15 \pm 0{\cdot}09$	$1{\cdot}8 \times 10^6 \pm 0{\cdot}49 \times 10^6$
pCF10 (*Ent. faecalis*)	54	>1000	yes	yes	no	$0{\cdot}29 \pm 0{\cdot}03$	$1{\cdot}2 \times 10^7 \pm 0{\cdot}7 \times 10^7$
pXO16 (*B. thuringiensis*)	200	>1000	no	no	no	$0{\cdot}042 \pm 0{\cdot}004$‖	$1{\cdot}6 \times 10^6 \pm 0{\cdot}7 \times 10^6$‖

* The number of cells in F-plasmid-mediated mating aggregates was estimated by Achtman (1975).

† Autoaggregation indicates whether the donor or recipient is able to form aggregates without the presence of the opposite mating type.

‡ Conjugation rate expressed as transconjugants produced per donor per minute, calculated using the nonlinear regression analysis described by Wilkinson (1961).

§ Recipient concentration (cells ml^{-1}) at which the conjugation rate is half its maximal value, calculated using the nonlinear regression analysis described by Wilkinson (1961).

‖ Data from Andrup *et al.* (1998) are included for comparison.

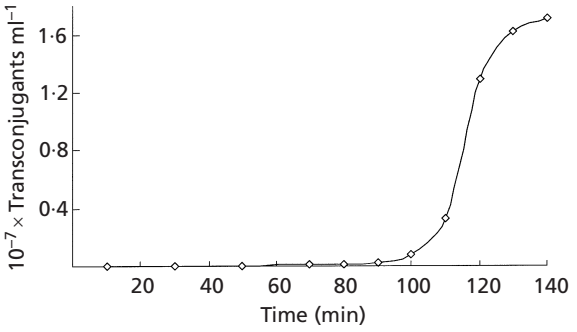

Fig. 3. Appearance of transconjugants in pCF10-mediated conjugation in *Ent. faecalis*. Donors ($9{\cdot}5 \times 10^4$ cells ml^{-1}) were combined with an excess of recipients ($1{\cdot}8 \times 10^7$ cells ml^{-1}) and the number of transconjugants is plotted as a function of time.

Ent. faecalis matings

Similar to the experiment with F-plasmid-mediated mating, we wanted to estimate the lag-period preceding the carrying out of donor activity by newly formed transconjugants. Donors ($9{\cdot}5 \times 10^4$ cells ml^{-1}) were combined with high concentrations of recipients ($1{\cdot}8 \times 10^7$ cells ml^{-1}) and the kinetics of transconjugant formation were determined. In Fig. 3, the number of transconjugants in the mating mixture is displayed as a function of time. A drastic increase in transconjugants, suggesting that transconjugants had become proficient donors, appeared after approximately 100 min. The transconjugant population reached a size similar to the initial number of donors after about 90 min, indicating that each donor, on average, had donated the plasmid once. The corresponding time for the F plasmid was approximately 10 min. Considering the pheromone-inducible nature of the conjugation system, it was likely that the induction of the donors with the relevant pheromone prior to mating would increase the efficiency of transfer and influence the maximal conjugation rate. Therefore the experiment was repeated with donors induced with pheromone prior to mating.

Estimation of maximal pheromone induction

To estimate the amount of pheromone necessary for maximal induction of the donors, a set of mating experiments with increasing volumes of pheromone-containing medium, prepared as described in Methods, was performed. Prior to the addition of recipient cells, the donors were incubated for 60 min in the presence of the pheromone medium. The cells were allowed to mate for 45 min, and the number of transconjugants formed ml^{-1} was determined as a function of the percentage of pheromone medium in the mating mixture. The number of donors and recipients at the start of the mating experiments was $1{\cdot}1 \times 10^7$ and $2{\cdot}1 \times 10^7$ cells ml^{-1}, respectively (determined in separate tubes without pheromone medium to avoid the formation of aggregates). The results suggested that if 20% or more of the mating mixture was pheromone medium, the donors were maximally induced for conjugation activity under the described culture conditions.

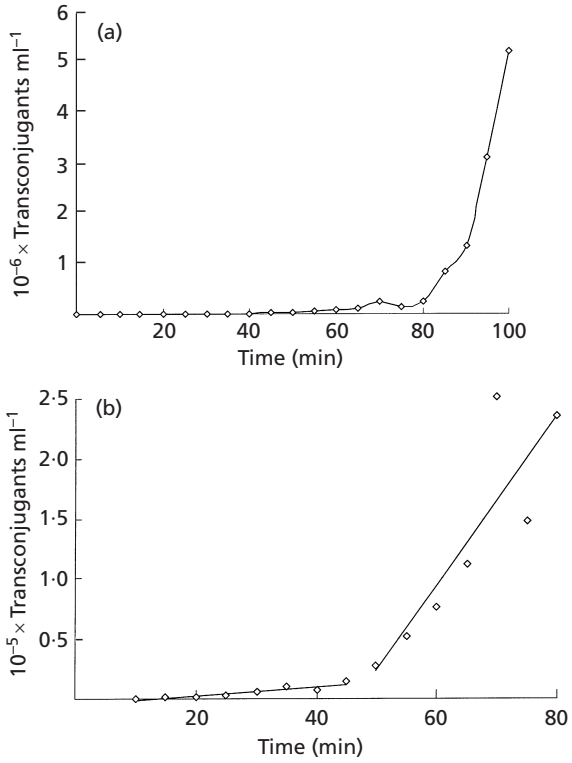

Fig. 4. Appearance of transconjugants in pCF10-mediated conjugation in *Ent. faecalis* as a function of time. The donors were induced with pheromone prior to mating. Donors ($1{\cdot}3 \times 10^4$ cells ml^{-1}) were combined with recipients ($8{\cdot}7 \times 10^6$ cells ml^{-1}) at time 0. (b) The first 80 min of the experiment presented in (a). The linear regression of the data is shown; $R^2 = 0{\cdot}89$, $\alpha = 363$ from 10 to 45 min and $R^2 = 0{\cdot}76$, $\alpha = 7084$ from 50 to 80 min. Transconjugants were selected on agar plates containing streptomycin and tetracycline.

Ent. faecalis matings with induced donors

In Fig. 4, the data from a mating experiment using pheromone induced donors are presented (the donors were incubated in 20% pheromone medium for 1 h prior to mating). An excess of recipients was added to optimize mating efficiency ($1{\cdot}3 \times 10^4$ donor cells ml^{-1} were combined with $8{\cdot}7 \times 10^6$ recipient cells ml^{-1}). In comparison with the transfer kinetics of uninduced donors (Fig. 3), the strong non-linear increase in transconjugants appeared about 20 min earlier (after 80 min). Also, a transconjugant population size similar to the initial number of donors ($1{\cdot}3 \times 10^3$ cells ml^{-1}) was reached after approximately 40 min, which is about half the time required by the uninduced donors (Fig. 3). In Fig. 4(b), the first 80 min of the mating experiment is shown. Apparently, the kinetics of plasmid transfer could be divided into two phases. In the first phase (from 10 to 45 min), the formation of transconjugants was fairly closely proportional to mating time (slope = 363;

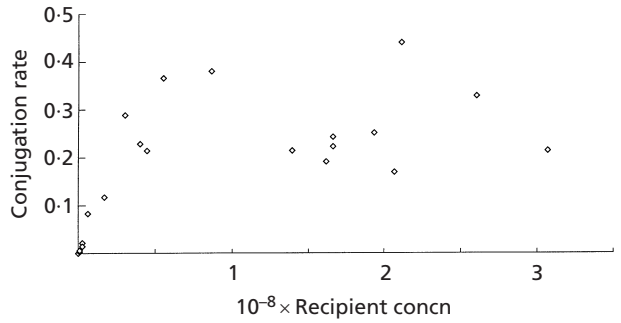

Fig. 5. Correlation between conjugation rate (transconjugants per donor per minute) and recipient concentration (c.f.u. ml^{-1}) in pCF10-mediated conjugation in *Ent. faecalis*. The donor concentration was $9{\cdot}8 \times 10^3 \pm 4{\cdot}0 \times 10^3$ c.f.u. ml^{-1} at the start of the matings. The recipient concentration was calculated using the method described in Methods.

$R^2 = 0{\cdot}89$). In the second phase (from 50 to 80 min), the proportionality was less pronounced; however, the rate of formation was significantly higher (slope = 7084; $R^2 = 0{\cdot}76$). From the measured generation time ($32{\cdot}8 \pm 2{\cdot}9$ min) the number of donors at $t = 27{\cdot}5$ and $t = 65$ min was calculated as $2{\cdot}2 \times 10^4$ and $4{\cdot}9 \times 10^4$, respectively. The conjugation rate was calculated as 0·016 transconjugants per donor per minute in the first phase and as 0·14 in the second phase, assuming that the transconjugants have not yet matured into proficient donors.

Correlation between recipient concentration and conjugation rate

To determine the correlation between conjugation rate and recipient concentration in pCF10-mediated conjugation in *Ent. faecalis*, a series of 23 mating experiments with initial recipient concentrations in the range of 1×10^3 to 3×10^8 cells ml^{-1} was conducted. The donor concentration was held relatively constant ($9{\cdot}8 \times 10^3 \pm 4{\cdot}0 \times 10^3$ cells ml^{-1}). The estimated conjugation rates determined as the number of transconjugants produced per donor per minute are plotted in Fig. 5 as a function of recipient concentration. Even though there was a large variance between the experiments, an upper limit to conjugation activity was observed. Using the mathematical model based on the resemblance between the kinetics of bacterial conjugation and enzyme reaction kinetics (Andrup *et al.*, 1998) the maximal conjugation rate (V_{max}) and the recipient concentration at which the conjugation rate was half its maximal value (K_m) were calculated. The results are presented in Table 1.

DISCUSSION

The analyses of only a few conjugation systems have included an estimation of basic kinetic parameters (Cullum *et al.*, 1978; Cullum & Broda, 1979; Dunny *et al.*, 1982; Andrup *et al.*, 1998). These kinetic parameters may include: (a) duration of a single mating event, (b) transconjugant maturation time, (c) the donor 'recovery period' between rounds of matings, (d) the distribution of conjugative activity among the donors, and (e) the possibility that a donor can transfer to more than one recipient at a time. We have recently suggested that the kinetics of conjugation may be described by a model analogous to the Michaelis–Menten model for enzyme catalysis in which the donor cell (the 'enzyme') transforms the recipient cell into a transconjugant without being altered itself (Andrup *et al.*, 1998). This allows a conjugation system to be characterized by two constants: the maximal conjugation rate, V_{max}, and K_m, which is the concentration of recipients that results in half the maximal conjugation rate.

V_{max} and K_m have been determined for the conjugation system encoded on plasmid pXO16 in *B. thuringiensis*. This conjugation system is characterized by the formation of large aggregates comprising interconnected donor and recipient cells in liquid medium (Andrup *et al.*, 1993, 1996; Jensen *et al.*, 1995). In the present study, we have analysed the kinetics of plasmid transfer in two conjugation systems (the F plasmid from *E. coli* and the pheromone-induced conjugation system encoded by pCF10 in *Ent. faecalis*), and compared them with the conjugation system in *B. thuringiensis*. These three conjugation systems employ different mechanisms for the establishment of mating pairs, the sizes of mating aggregates are different, and they have different host ranges. Also, the sizes of the plasmids are markedly different, plasmids pCF10, F and pXO16 being approximately 54, 100 and 200 kb, respectively. However, these conjugation systems have some characteristics in common: they are able to sustain conjugative transfer in liquid medium and the transfer efficiencies are very high (frequencies close to 100% are often reached).

Our results are consistent with an ability of newly formed transconjugants in all three conjugation systems to function as donors only after a maturation time of 40–80 min. This is in agreement with the findings of Cullum *et al.* (1978) for F-plasmid-mediated conjugation in *E. coli*. Assuming that plasmid transfer in *B. thuringiensis* and *Ent. faecalis* proceeds via a single-strand transfer mechanism as in *E. coli* (Pansegrau & Lanka, 1996), maturation of transconjugants requires the synthesis of the complementary DNA strand, aggregation substances, and probably other, as yet uncharacterized, transfer functions. The transfer functions encoded on pXO16 and F are permanently derepressed, whereas full transfer competence in pCF10-mediated conjugation required prior induction of the donors with the relevant pheromone.

Previous research suggests both for the F plasmid in *E. coli* (Cullum *et al.*, 1978) and for pXO16-mediated conjugation in *B. thuringiensis* (Andrup *et al.*, 1998) that conjugation activity is not confined to a subpopulation of very potent donors, but is more or less equally distributed among the donors. Likewise, the donors in both systems require a lag period between rounds of transfer. A duration of 30 min has previously been reported for this lag period between rounds of transfer

in F-plasmid-mediated transfer in *E. coli* (Cullum *et al*., 1978). Here, we report a maximal conjugation rate of about 0·15 transconjugants per donor per minute. As the variance of conjugation activity among the donors has been shown to be small, this suggests a transfer event every 7 min on average. Assuming a speed of DNA transfer similar to Hfr matings and conjugation in *B. thuringiensis* of about 1 kb s^{-1}, this only allows for a lag period of about 5 min. In our mating experiments, we ensured that the donor activity was maximal, i.e. the recipient concentration was not a limiting factor. The differences between our results and those obtained by Cullum *et al*. (1978) may be related to the ratio of donors to recipients [we find maximal conjugation rate at a recipient to donor ratio of 4×10^3 whereas the ratio used by Cullum *et al*. (1978) was approximately 10^2] and the mating conditions. We have previously determined the lag period between rounds of transfer in pXO16 conjugation to be about 10 min in *B. thuringiensis* (Andrup *et al*., 1998). In pCF10-mediated mating we estimate the maximal conjugation rate, using pheromone-induced donors, to be 0·29 transconjugants per donor per minute. This corresponds to a mating event every 3 to 4 min, allowing only for a few minutes between rounds of transfer.

A series of mating experiments using different recipient concentrations, and maintaining a relatively constant donor concentration, was conducted and a 'saturation' of the donors was observed in both conjugation systems at high recipient concentrations, meaning that the donors were displaying maximal conjugation activity. This maximal conjugation rate was calculated, assuming that the kinetics can be described by a model analogous to the Michaelis–Menten model for enzyme catalysis, at 0·15 transconjugants per donor per minute in *E. coli* (F plasmid) and 0·29 transconjugants per donor per minute in *Ent. faecalis* (pCF10). These are relatively high values in comparison with pXO16-mediated conjugation, where V_{max} has been determined to be 0·05 transconjugants per donor per minute (Andrup *et al*., 1998). This may be related to the sizes of the plasmids. Interestingly, the conjugation rate was found to be inversely proportional to plasmid size in these three conjugation systems and the actual speed of DNA transfer may be constant and close to the speed of DNA replication.

One obstacle in analysing living micro-organisms is the large variability between experiments. We believe that these types of experiments may be sensitive to small variations in the physical and chemical conditions, but also to physiological conditions such as growth phase. Measuring the conjugation rate at different recipient concentrations is quite demanding, as many conjugation experiments are required. Knowing the recipient concentration above which the donors are saturated enables the conjugation rate to be estimated from a single mating experiment. Assuming that conjugation activity is evenly distributed among the donors, which has been shown for pXO16-mediated conjugation in *B. thuringiensis*, and that each donor cell is able to transfer the plasmid to only one recipient at a time, the conjugation rate can be calculated as the increase in transconjugants per donor per minute in a mating experiment with a large excess of recipients. It is important to sample the transconjugants early in mating (e.g. after 30 and 45 min) to ensure that newly formed transconjugants have not yet matured into proficient donors. In the estimation of conjugation rate for pCF10-mediated transfer (Fig. 4b) we observed that the excess of recipients was not sufficient to reach the maximal conjugation rate. In Fig. 5, it is seen that a recipient concentration above 1×10^8 ensured saturating conjugation conditions. In contrast, in the *E. coli* experiment presented in Fig. 1(b), we were able to estimate a conjugation rate for the F plasmid conjugation system which was quite similar to the one determined using the Michaelis–Menten model (0·14 versus 0·15 transconjugants per donor per minute).

Estimating the K_m value revealed fundamental differences among the conjugation systems. In the case of the F plasmid, the sex-pili of the donor cell reach 1–2 μm from the cell surface (Ippen-Ihler & Minkley, 1986), enabling them to detect and entrap the recipient cell efficiently. This is reflected, along with differences between the affinity of the 'adhesins' of the donor and the corresponding 'ligands' of the recipient, in the values of K_m in the three conjugation systems (Table 1).

It can not be ruled out that other mathematical correlations than the Michaelis–Menten equation may describe the phenomenon of bacterial conjugation more accurately and it is probably too ambitious to generalize about transfer kinetics given the profound differences in conjugation systems. In particular, the kinetics of conjugative transfer of pCF10 seems to deviate from the Michaelis–Menten correlation. This may not be surprising in view of the physiology of mating pair formation in *Ent. faecalis*. When the donors are induced by pheromones they are able to form aggregates with other donor cells, and in contrast to F plasmid- and pXO16-mediated conjugation, this may produce non-productive mating pairs and the access to some cells may be physically blocked. However, the kinetics of plasmid transfer mediated by pCF10 adheres to the basic criteria stipulated by the equation, having an upper limit of conjugation rate and showing linearity between conjugation rate and recipient concentration at the low range of recipient concentration.

Analysing different conjugation systems constantly adds to our knowledge of variations of the theme of horizontal DNA transfer. From being an exotic peculiarity of bacterial recombination it is now recognized as a widespread phenomenon, whose implications with regard to evolution and dissemination of genetic elements have just begun to be understood. Very recently a replicon from a conjugative plasmid, pAW63 (Wilcks *et al*., 1998), from the commercially used *B. thuringiensis* subsp. *kurstaki* HD73 was found to be nearly identical to one of the virulence plasmids from the highly pathogenic *Bacillus anthracis* (Wilcks *et al*., 1999). The

fundamental differences in conjugative mechanisms with respect to requirement for solid surfaces, induction of aggregate formation, repression, and abilities to mobilize nonconjugative plasmids, etc., stress the importance of collecting data on as many aspects as possible. When characterizing conjugation, it is not sufficient to focus on a particular species or group of bacteria, as many new systems probably remain to be discovered. The observed conjugation between Gram-positive and Gram-negative bacteria as an example of the broad host range and variability of conjugation systems (Trieu-Cuot *et al*., 1987, 1988, 1993), and the ability of the pXO16-encoded conjugation system in *B. thuringiensis* to mobilize nonconjugative plasmids not containing *mob* genes and *oriT* sites, known to be required in other conjugation systems (Andrup *et al*., 1996), both highlight the importance of analysing individual systems and taking care in generalizing from one system to another.

ACKNOWLEDGEMENTS

We thank Garry Dunny and Per Amstrup Pedersen for providing the strains, Gert B. Jensen for critically reading the manuscript, and Janet Stewart for linguistic assistance. Bjørn Andersen Nexø is acknowledged for programming the computer software used for calculations of K_m and V_{max}.

This work was supported by grants from The Carlsberg Foundation, Løvens Kemiske Fabriks Forskningsfond and Beckett-Fonden.

REFERENCES

Achtman, M. (1975). Mating aggregates in *Escherichia coli* conjugation. *J Bacteriol* **123**, 505–515.

Andrup, L., Damgaard, J. & Wassermann, K. (1993). Mobilization of small plasmids in *Bacillus thuringiensis* subsp. *israelensis* is accompanied by specific aggregation. *J Bacteriol* **175**, 6530–6536.

Andrup, L., Jørgensen, O., Wilcks, A., Smidt, L. & Jensen, G. B. (1996). Mobilization of ''non-mobilizable'' plasmids by the aggregation-mediated conjugation system of *Bacillus thuringiensis*. *Plasmid* **36**, 75–85.

Andrup, L., Smidt, L., Andersen, K. & Boe, L. (1998). Kinetics of conjugative transfer: a study of the plasmid pXO16 from *Bacillus thuringiensis* subsp. *israelensis*. *Plasmid* **40**, 30–43.

Bullock, W. O., Fernandez, J. M. & Short, J. M. (1987). XL1-Blue: a high efficiency plasmid transforming *recA Escherichia coli* strain with beta-galactosidase selection. *BioTechniques* **5**, 376–378.

Casadaban, M. J. & Cohen, S. N. (1980). Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. *J Mol Biol* **138**, 179–207.

Clewell, D. B. (1993a). Bacterial sex pheromone-induced plasmid transfer. *Cell* **73**, 9–12.

Clewell, D. B. (1993b). *Bacterial Conjugation*. New York: Plenum.

Cullum, J. & Broda, P. (1979). Recipient competence in F′*lac* matings of *Escherichia coli* K12. *J Bacteriol* **137**, 281–284.

Cullum, J., Collins, J. F. & Broda, P. (1978). Factors affecting the kinetics of progeny formation with F′*lac* in *Escherichia coli* K12. *Plasmid* **1**, 536–544.

Dunny, G. M., Brown, B. L. & Clewell, D. B. (1978). Induced cell aggregation and mating in *Streptococcus faecalis*: evidence for a bacterial sex pheromone. *Proc Natl Acad Sci USA* **75**, 3479–3483.

Dunny, G. M., Craig, R. A., Carron, R. L. & Clewell, D. B. (1979). Plasmid transfer in *Streptococcus faecalis*: production of multiple sex pheromones by recipients. *Plasmid* **2**, 454–465.

Dunny, G., Yuhasz, M. & Ehrenfeld, E. (1982). Genetic and physiological analysis of conjugation in *Streptococcus faecalis*. *J Bacteriol* **151**, 855–859.

Gasson, M. J., Swindell, S., Maeda, S. & Dodd, H. M. (1992). Molecular rearrangement of lactose plasmid DNA associated with high-frequency transfer and cell aggregation in *Lactococcus lactis* 712. *Mol Microbiol* **6**, 3213–3223.

Ippen-Ihler, K. A. & Minkley, E. G., Jr (1986). The conjugation system of F, the fertility factor of *Escherichia coli*. *Annu Rev Genet* **20**, 593–624.

Jarrett, P. & Stephenson, M. (1990). Plasmid transfer between strains of *Bacillus thuringiensis* infecting *Galleria mellonella* and *Spodoptera littoralis*. *Appl Environ Microbiol* **56**, 1608–1614.

Jensen, G. B., Wilcks, A., Petersen, S. S., Damgaard, J., Baum, J. A. & Andrup, L. (1995). The genetic basis of the aggregation system in *Bacillus thuringiensis* subsp. *israelensis* is located on the large conjugative plasmid pXO16. *J Bacteriol* **177**, 2914–2917.

Jensen, G. B., Andrup, L., Wilcks, A., Smidt, L. & Poulsen, O. M. (1996). The aggregation-mediated conjugation system of *Bacillus thuringiensis* subsp. *israelensis*: host range and kinetics. *Curr Microbiol* **33**, 228–236.

van der Lelie, D., Chavarri, F., Venema, G. & Gasson, M. J. (1991). Identification of a new genetic determinant for cell aggregation associated with lactose plasmid transfer in *Lactococcus lactis*. *Appl Environ Microbiol* **57**, 201–206.

Pansegrau, W. & Lanka, E. (1996). Enzymology of DNA transfer by conjugative mechanisms. *Prog Nucleic Acid Res Mol Biol* **54**, 197–251.

Salyers, A. A. (1993). Gene transfer in the mammalian intestinal tract. *Bio/Technology* **4**, 294–298.

Salyers, A. A., Shoemaker, N. B., Stevens, A. M. & Li, L. Y. (1995). Conjugative transposons: an unusual and diverse set of integrated gene transfer elements. *Microbiol Rev* **59**, 579–590.

Schmidt, T., Friehs, K. & Flaschel, E. (1996). Rapid determination of plasmid copy number. *J Biotechnol* **49**, 219–229.

Trieu-Cuot, P., Carlier, C., Martin, P. & Courvalin, P. (1987). Plasmid transfer by conjugation from *Escherichia coli* to gram-positive bacteria. *FEMS Microbiol Lett* **48**, 289–294.

Trieu-Cuot, P., Carlier, C. & Courvalin, P. (1988). Conjugative plasmid transfer from *Enterococcus faecalis* to *Escherichia coli*. *J Bacteriol* **170**, 4388–4391.

Trieu-Cuot, P., Derlot, E. & Courvalin, P. (1993). Enhanced conjugative transfer of plasmid DNA from *Escherichia coli* to *Staphylococcus aureus* and *Listeria monocytogenes*. *FEMS Microbiol Lett* **109**, 19–24.

Wilcks, A., Jayaswal, N., Lereclus, D. & Andrup, L. (1998). Characterization of plasmid pAW63, a second self-transmissible plasmid in *Bacillus thuringiensis* subspecies *kurstaki* HD-73. *Microbiology* **144**, 1263–1270.

Wilcks, A., Smidt, L., Økstad, O. A., Kolstø, A.-B., Mahillon, J. & Andrup, L. (1999). Replication mechanism and sequence analysis of the replicon of pAW63, a conjugative plasmid from *Bacillus thuringiensis*. *J Bacteriol* **181**, 3193–3200.

Wilkinson, G. N. (1961). Statistical estimations in enzyme kinetics. *Biochem J* **80**, 324–332.

Wirth, R. (1994). The sex pheromone system of *Enterococcus*

faecalis – more than just a plasmid-collection mechanism? *Eur J Biochem* **222**, 235–246.

Yin, X. M. & Stotzky, G. (1997). Gene transfer among bacteria in natural environments. *Adv Appl Microbiol* **45**, 153–212.

Zatyka, M. & Thomas, C. M. (1998). Control of genes for conjugative transfer of plasmids and other mobile elements. *FEMS Microbiol Rev* **21**, 291–319.

Received 8 January 1999; revised 16 April 1999; accepted 23 April 1999.