Plasmids can transfer to *Clostridium difficile* CD37 and 630Δ*erm* both by a DNase resistant conjugation-like mechanism and a DNase sensitive mechanism

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Abstract

Broad host range conjugative plasmids that replicate in *Escherichia coli* have been widely used to mobilise smaller replicons, bearing their cognate origin of transfer (*oriT*) into a variety of organisms that are less tractable genetically, such as *Clostridium (Clostridioides) difficile*. In this work we demonstrated that the *oriT* region of pMTL9301 (derived from RK2) is not required for transfer between *E. coli* and *C. difficile* strains 630Δ*erm* and CD37 and that this *oriT*-independent transfer is abolished in the presence of DNase when CD37 is the recipient. Transfer to the 630Δ*erm* strain is DNase resistant even without an obvious *oriT,* when *E. coli* CA434 is used as a donor and is sensitive to DNase when *E. coli* HB101 is the donor.

Introduction

Horizontal gene transfer (HGT) in bacteria is responsible for their enormous genome plasticity. This contributes to the rapid evolution of these organisms and to the current high profile problem of antibiotic resistance. There are thought to be three main mechanisms of HGT, transformation in which naked DNA is taken up by the recipient organism, transduction in which bacteriophages (phages) are responsible for the transfer of bacterial DNA from a donor to a recipient and conjugation in which specialised genetic elements mediate the spread of DNA from a donor to a recipient (for recent reviews see Soucy *et al.,* 2015; von Wintersdorff *et al.,* 2016).

Bacteria employ a diverse range of conjugation systems but most of these are based on a type 4 secretion system (T4SS) which mediates the transfer of DNA from donor to recipient. T4SSs are typically multi-protein systems. The first step is nicking the DNA to be transferred at a *cis* acting origin of transfer *oriT* then translocation of single stranded DNA from donor to recipient. In order to survive in the recipient the transferred DNA has to recombine into the recipient's genome via homologous recombination or by a genetic element encoded recombinase or transposase. Alternatively it can replicate separately from the host genome as a plasmid (for a recent review see Goessweiner-Mohr *et al.,* 2014). Integrated plasmids, conjugative transposons and integrative conjugative elements (ICE) can also mediate the transfer of the bacterial chromosome (Dordet-Frisoni *et al.,* 2014; Hochhut *et al.,* 2000; Wollman *et al.,* 1956). These genetic elements all encode a T4SS. A number of other conjugation systems exist which do not fit the more well studied T4SS mediated transfer from donor to recipient. In the Actinomycetales, double stranded DNA is transferred and the conjugation system resembles the segregation of bacterial chromosomal DNA in cell division and sporulation (Thoma and Muth 2012). However in all conjugation systems DNA is always contained within the cell(s) and the transfer process is consequently resistant to DNase.

In contrast to conjugation in transformation the recipient takes up DNA from its environment and incorporates this into its genome. Bacteria that are capable of natural transformation encode the proteins required for this process (reviewed in Soucy *et al.,* 2015) which is typically sensitive to DNase.

Clostridium difficile now called *Clostridioides difficile* has been shown to have a highly plastic genome with nearly a third consisting of mobile genetic elements (MGE) (Sebaihia *et al.*, 2006). These elements are mostly ICE and previous work from this laboratory has shown that these are capable of transfer between *C. difficile* strains and in some cases to other members of the Firmicutes such as *Bacillus subtilis* and *Enterococcus* spp. (Brouwer *et al.,* 2011; Jasni *et al.,* 2010; Mullany *et al.,* 1990).

The major virulence factors of *C. difficile* are encoded by a region of the genome called the PaLoc which as well as genes encoding two potent toxins (A and B) also contains genes that encode positive and negative regulators of toxin gene expression and also possibly toxin export (Braun *et al.,* 1996). This region is also capable of transfer to non-toxigenic strains on large genomic fragments (Brouwer *et al.,* 2013). PaLoc transfer was resistant to DNase so was presumably mediated by conjugation.

One of the reasons that gene transfer in *C. difficile* was investigated was to develop genetic tools to study this important pathogen. Conjugative transposons were the first genetic elements used to investigate *C. difficile* genetics (reviewed in Mullany *et al.,* 2015). Initially the conjugative transposon Tn*916* was used for gene cloning. Genes were introduced into Tn*916* in *B. subtilis* and transferred from this host to *C. difficile* (Mullany *et al.,* 1994). Subsequently a plasmid based system was developed where small shuttle plasmids could be transferred from *Escherichia coli* to *C. difficile* (Purdy *et al.,* 2002). One of these, pMTL9301 has been used extensively to transfer genes from *E. coli* to *C. difficile* (Purdy *et al.,* 2002). The means of transfer had been assumed to be by conjugation as all matings were from an *E. coli* host which contained a broad host range IncP plasmid R702 (which encodes a T4SS) to mobilise pMTL9301 into *C. difficile.* The latter plasmid has been engineered to contain *E. coli* and *C. difficile* origins of replication and an *oriT* that is recognised by the R702 conjugation system (Purdy *et al.,* 2002). However formal proof that the primary means of transfer is conjugation, i.e. DNase resistant transfer, has never been reported. Likewise formal proof that Tn*916* and Tn*5397* transfer by conjugation from *B. subtilis* has not been reported. In this paper we show that pMTL9301 transfers by a DNase sensitive mechanism and a DNase resistant conjugation-like mechanism but that Tn*916* and its close relative Tn*5397* only transfer by conjugation.

2 Materials and Methods

Strains, plasmids and culture conditions. All the bacterial strains and plasmids used in this study are shown in table 1. *E. coli* strains carrying plasmids were grown in Luria-Bertani (LB) medium at 37℃. *C. difficile* and *B. subtilis* strains were grown in brain heart infusion (BHI) agar or broth (Oxoid Ltd, Basingstoke, UK) the former supplemented with 5 % defibrinated horse blood (E and O Laboratories) and incubated in an anaerobic atmosphere (80 % nitrogen, 10 % hydrogen and 10 % carbon dioxide) or aerobically.

Media were supplemented when required with antibiotics at the following concentrations: erythromycin 400 µg/ml or 10 µg/ml, rifampicin 25 µg/ml and tetracycline 10 µg/ml. When specified, Deoxyribonuclease I (DNase) from bovine pancreas (Sigma- Aldrich) was added to the mating mix to a final concentration of 50 µg/ml.

Vector construction. pMTL9301Δ*oriT* was constructed by removing the *oriT*-containing 700 bp *Eco*RI fragment from pMTL9301. After digestion with *Eco*RI the plasmid was incubated with T4 DNA ligase, self-ligated and transformed into *E. coli* DH5α. To confirm that the *oriT* region has been deleted the plasmid was subject to restriction digests and PCR amplification (using primers flanking the *oriT* region [table 2]). Deletions were confirmed by DNA sequencing.

Gene transfer procedures. All manipulations involving clostridial strains were undertaken anaerobically in a Don Whitley Mk II Anaerobic Workstation. *E. coli* was transformed as previously described (Sambrook *et al*., 1989). For gene transfer experiments from *E. coli* CA434 or HB101 to *C. difficile* the method described by Purdy *et al.,* (2002) was followed. In brief bacteria were mixed in a 1:1 ratio on antibiotic free plates and after 24 hours incubation at 37 degrees in an anaerobic atmosphere were re-suspended in 1 ml of BHI broth then spread on selective plates. Transconjugants/transformants appeared after 72 hours. In some cases DNase (50 µg/ml) was added to the mating mix. *C. difficile* containing plasmids were selected by plating on *C. difficile* selective supplement D-cycloserine (0.25 mg/ml), and cefoxitin (0.008 mg/ml) (to select against *E. coli*) and erythromycin (10 µg/ml) to select for plasmid transfer. In some experiments the *E. coli* donor was killed by heating at 85°C for 30 minutes on a heating block. To prove that the mobile genetic element under investigation had transferred to *C. difficile* all putative transconjugants were sub-cultured anaerobically and aerobically (the latter to confirm there are no surviving donor cells) and in some cases the *toxB* gene, or the region flanking the PaLoc (this confirms that the transconjugants are *C. difficile,* strain 630Δ*erm* or strain CD37 respectively) was amplified and sequenced.

When *B. subtilis* was used as the donor, donors and recipients were mixed on 0.45 µm pore size cellulose nitrate filters (Sartorius, Epsom, UK) on BHI agar plates for 24 hours prior to plating on selective agar, using a method previously described (Brouwer *et al.,* 2013). Transconjugants were selected on agar containing tetracycline (10 µg/ml) to select for the conjugative transposons Tn*916* or Tn*5397* and *C. difficile* selective supplement D-cycloserine (0.25 mg/ml), and cefoxitin (0.008 mg/ml) to select against *B. subtilis.* Where specified, DNase was added to the mating mix.

To test for the ability of purified plasmid to transform *C. difficile,* plasmid DNA (final concentration of 4 µg/ml) was mixed with *C. difficile* on plates as described above for matings between *E. coli* and *C. difficile* except in this case plasmid DNA replaced *E. coli.* In some cases *E. coli* cell extracts were prepared and added.

Molecular biology techniques. Plasmid DNA was isolated from *E. coli* strains using the QIAprep Spin Miniprep kit (Qiagen, UK). Plasmid DNA from *C. difficile* was isolated by making whole genome DNA preparations (using the Puregene Yeast/Bact.kitB, Qiagen, UK) then using this to transform *E. coli* DH5α. Plasmids were subsequently isolated from this strain as described above. PCR amplification was carried out using the NEB Taq polymerase kit (New England Biolabs, UK) according to the manufacturer’s instructions. All primers used in this work are shown in table 2.

3 Results

3.1 DNase treatment reduces the transfer frequency of a shuttle plasmid from E. coli *CA434 to* C. difficile *strain CD37 by 5 orders of magnitude but has no effect on the transfer frequency to* C. difficile *630Δerm*

Plasmid pMTL9301 was transferred from *E. coli* CA434 to *C. difficile* CD37 at a frequency of around 3.26 x 10^{-5} transconjugants per donor and to *C. difficile* 630Δ*erm* at a frequency of around 3 x 10^{-5} transconjugants per donor (table 3) similar to previously reported transfer frequencies (Purdy *et al.,* 2002). Incorporation of DNase into the mating mix prior to plating onto selective agar, resulted in the frequency of transfer decreasing by 5 orders of magnitude to around 10^{-10} transconjugants per donor for CD37 but no change in transfer frequency was observed when 630Δ*erm* was used as the recipient (table 3).

3.2 Deletion of the oriT *from pMTL9301 does not abolish transfer from* E. coli *to* C. difficile *but does abolish transfer to CD37 in the presence of DNase*

The fact that pMTL9301 transfer to CD37 is drastically reduced in the presence of DNase indicates that as well as transferring by conjugation the plasmid ~~is~~ may also be entering *C. difficile* by a transformation like mechanism. To investigate this further we deleted the *oriT* region from pMTL9301 to generate pMTL9301Δ*oriT*. This plasmid transferred from *E. coli* CA434 in the absence of DNase to CD37 and 630Δ*erm* (although at much lower frequencies than observed with pMTL9301) (table 3). No transfer (the detection limit was < 10^{-10} transconjugants per donor or recipient) was observed to CD37 when DNase was included in the medium, although transfer to 630Δ*erm* was still observed (table 3). That pMTL9301 and pMTL9301Δ*oriT* were indeed transferred to *C. difficile* CD37 and 630Δ*erm* was confirmed by preparing plasmids from representative transconjugants and demonstrating that they contain either intact *oriT* (pMTL9301) or had this region deleted in the case of transconjugants containing pMTL9301Δ*oriT* (fig 1). Plasmid structure was verified by DNA sequencing and restriction mapping (data not shown). That the transconjugants were the expected *C. difficile* strain and no *E. coli* were present was confirmed by PCR and sequencing (as described in the methods, results not shown) and confirming that there is no growth after 48 hours aerobic incubation respectively.

*3.3 pMTL9301 and pMTL9301Δ*oriT *can be transferred from* E. coli *HB101 to* C. difficile *CD37 and 630Δerm only in the absence of DNase*

According to Purdy *et al.,* (2002) CA434 was made by transferring the conjugative plasmid R702 into HB101. Therefore to test the role of R702 in transfer of pMTL9301 and pMTL9301Δ*oriT* HB101 was used as a donor. Plasmid pMTL9301 transferred from this host to 630Δ*erm* at a frequency of around 10^{-9} per recipient and 10^{-10} per donor and at a frequency of 10^{-9} per recipient and 10^{-10} per donor to CD37 (table 3). When HB101 containing pMTL9301Δ*oriT* was used as the donor and 630Δ*erm* the recipient transconjugants appeared at a similar frequency but no transconjugants were observed when CD37 was the recipient. When DNase was incorporated in the media no transconjugants were obtained in any of the above combinations of recipients and donors. The detection limit in these experiments was < 10^{-10} transconjugants per donor or recipient. That transconjugants/transformants were genuine was confirmed as described above and in the methods section.

3.4 The transfer process requires live donor and no transfer is observed when plasmid DNA only is used in the medium.

We incubated *C. difficile* with pMTL9301 as outlined in the methods section. Erythromycin resistant transformants were never obtained in these experiments. No erythromycin resistant transformants were obtained when heat killed *E. coli* containing wild-type plasmids were used as donors (table 3).

*3.5 Transfer of Tn*5397 *and Tn*916 *from* Bacillus subtilis *is not affected by DNase treatment*

In order to test if the DNase sensitive transfer is a more general phenomenon we examined the transfer of Tn*5397* and Tn*916* (these genetic elements both encode resistance to tetracycline by the *tet*(M) gene - reviewed in Mullany *et al.,* 2015) from *B. subtilis* to *C. difficile* CD37. Tn*916* and Tn*5397* containing transconjugants were obtained at a frequency of around 1 x10^{-7} and 1x10^{-8} transconjugants per donor respectively, very similar to previously reported transfer frequencies for these elements (Mullany *et al.,* 1990; 1994). This was the same in both the presence and absence of DNase (results not shown).

4 Discussion

The results show that *C. difficile* CD37 can take up plasmid DNA by at least two ways, a DNase resistant conjugation-like mechanism and a DNase sensitive mechanism. However the latter is not like most previously described transformation mechanisms in that naked DNA is not sufficient for the transfer process and live donor cells are required. It appears that in plate mixtures of *E. coli* CA434 and *C. difficile* CD37 both DNase resistant and sensitive transfer occur simultaneously, but at different frequencies, as DNase treatment drastically reduces but does not completely abolish plasmid transfer. Likewise deletion of *oriT* reduces plasmid transfer frequencies but does not stop it; low frequency plasmid transfer to CD37 becomes completely sensitive to DNase. A similar DNase sensitive transfer of DNA has been observed when *C. difficile* donors containing Tn*6194* were mixed with a recipient strain (Wasels *et al.,* 2015). In this experiment cell-cell contact was required and free DNA did not result in transfer of Tn*6194* (Wasels *et al.,* 2015). The role of the *oriT* in transfer in these experiments was not investigated.

We also demonstrated that the *oriT mob* region is not required for transfer of plasmid pMTL9301Δ*oriT* from *E. coli* CA434 to *C. difficile* 630Δ*erm* but that transfer in this case is resistant to DNase. Work by Lee *et al*., (2012) has shown that plasmids that do not have obvious origins of transfer can be mobilised by ICE*BS1* and hypothesised that the coupling protein encoded by ICE*BS1* is recruiting the replicative relaxosome to the conjugation machinery encoded by the ICE. It is possible that a similar interaction is being mediated by R702 with pMTL9301Δ*oriT*. This observation implies that the interaction of conjugative elements with replicative as well as conjugative relaxases may be common in nature.

In this work we also showed that transfer of Tn*5397* and Tn*916* from *B. subtilis* is completely resistant to DNase treatment. We have previously shown that transfer of the PaLoc is also DNase resistant (Brouwer *et al.,* 2013), further demonstrating that *C. difficile* can acquire DNA by at least two different pathways. More work is required to determine why transfer of some genetic elements is sensitive to DNase whereas the transfer of other genetic elements is not, but this is likely to depend on both the elements themselves and the donor and recipient strains. Work by Wang *et al.,* (2007) has shown that a non-conjugative plasmid can be transferred from *E. coli* HB101 to *B. subtilis* in a DNase sensitive manner but that transfer was not always completely stopped by DNase. They suggested that DNA could be protected from DNase on solid agar. It is also possible that the partial *tra* operon in HB101 (see below) provided a mating bridge that was permeable to DNase.

To further investigate the phenomenon and to determine if plasmid R702 is required for the transfer process we found that both pMTL9301 and pMTL9301Δ*oriT* can be transferred into both CD37 and 630Δ*erm* from *E. coli* HB101 (the parent of CA434 Purdy *et al.,* 2002), although at a much lower frequency than when CA434 is the donor (table3). As transfer from HB101 is completely sensitive to DNase and does not require an *oriT* (there is no difference in the frequency of transfer of pMTL9301 and pMTL9301Δ*oriT*) this indicates that a transformation like mechanism may being used to transfer the plasmid from *E. coli* HB101 to *C. difficile.* Furthermore, the fact that the transfer frequency is much lower from HB101 than from CA434 indicates that transfer is via a different mechanism in the two strains. A search of the available HB101 genome https://www.ncbi.nlm.nih.gov/nuccore/CP011113 (Jeong *et al.,* 2017) does show that this strain contains some *tra* genes although not all the genes required for conjugation are present, importantly no TraF-encoding gene or members of the *trb* operon could be found. These gene products are thought to be required for stable mating pair formation and encode proteins required for mating bridge formation (reviewed in Zatyka and Thomas 1998). It is possible that the *tra* genes that remain in HB101 can mediate the formation of a mating pair (and that the mating bridge is permeable to DNase) but with much less efficiency than a whole R702.

An alternative possibility is that the transfer we are observing is via a transformation-like mechanism for example in *Thermus* spp it has been shown that mutation of some of the genes involved in natural transformation stops DNA transfer in a DNase resistant yet *oriT* independent transfer system (Blesa *et al.,* 2015). These workers proposed that the donor cell was pushing the DNA from the donor in a way analogous to conjugation and that the recipient was pulling the DNA into the cell using the competence system (Blesa *et al.,* 2015). It is possible that something similar is happening in the transfer system we are observing in *C. difficile*; perhaps the donor is secreting DNA or a sub-population is lysing and the *C. difficile* “competence system” then “pulls” the DNA into the recipient cell. The reason that naked DNA alone is not taken up by the recipient could be that the competence system requires signals produced by the donor in order to be expressed. This would explain the requirement for viable donor *E. coli.* A potentially similar system has been observed in gene transfer between *E. coli* strains in which a continual supply of plasmid derived from live donor cells was required to transform recipients (Etchuuya *et al.,* 2011). This system was termed cell to cell transformation. Another similar system is the transfer of a shuttle plasmid from *E. coli* to *B. subtilis* on agar surfaces. In this case transfer was DNase sensitive but required intimate contact between the donor and recipient strains (Wang *et al.,* 2007). These authors postulated that that *E. coli* was stimulating a competence system in *B. subtilis.*

The work reported in the current study shows that *C. difficile* has a remarkable ability to obtain new DNA. The unexpected observation that it can take up plasmid DNA from an unrelated organism (*E. coli*) without a complete conjugation system or a *cis* acting *oriT* (although *oriV* may be able to substitute for this in some instances) indicates that the organism has the potential to acquire almost any DNA sequence. Presumably the only limiting factors are the ability for the incoming DNA to be able to replicate or be incorporated into the host chromosome.

Our study also has implications for the containment of genetically modified organisms, as we have shown that non-conjugative non-mobilisable plasmids can still be taken up by an organism that was previously thought not to be naturally competent and it is important to determine how common this phenomenon is in nature. Furthermore, an intact *oriT* is not required for transfer. We plan to undertake a detailed molecular analysis of this process.

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