Characterization of a “Mobilization Cassette” in Transposon Tn4399 from *Bacteroides fragilis*

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Derivatives of nonconjugal plasmids that carry Tn4399, a transposon isolated from *Bacteroides fragilis*, can be mobilized for transfer by the broad-host-range IncP plasmids pRK231 or R751 in *Escherichia coli*. To characterize regions of Tn4399 involved in mobilization, we have isolated and analyzed subcloned fragments of Tn4399 in *E. coli*, as well as mutations within the element. We have identified a “mobilization cassette” within a 2.8-kb region of Tn4399 which, when cloned into mobilization-deficient plasmids, allows these plasmids to be mobilized *in trans* by the IncP plasmids pRK231 and R751. The 2.8-kb region has been sequenced, and several open reading frames have been identified. Mutants defective in two genes, designated *mocA* and *mocB*, coding for deduced products of 36.4 and 16.4 kDa, respectively, cannot be mobilized by either IncP plasmid; these mutants can be complemented in the presence of the respective wild-type genes *in trans*. This suggests that the putative MocA and MocB proteins have a role in the mobilization process. The 36.4-kDa MocA protein contains a 14-amino-acid sequence which is closely related to a highly conserved motif within DNA relaxases encoded by a wide variety of conjugal or mobilizable plasmids. Subcloning experiments also lead to the localization of an *oriT* region within a 199-bp fragment, internal to the mobilization cassette.

Bacterial conjugation is a process in which plasmid or chromosomal DNA is transferred from a bacterial donor cell to a suitable recipient cell. As a result, genetic information is disseminated to a wide variety of bacterial species (3). A bacterial conjugal element or transfer factor that is capable of self-transfer must contain a *cis*-acting origin of transfer, *oriT*, and in addition encode and regulate numerous *trans*-acting transfer functions in order to establish effective contacts with recipient cells and to initiate DNA transfer and replication (3). Nonconjugal plasmids which contain an *oriT* site but not the necessary transfer functions may be efficiently mobilized if a transfer factor which encodes the essential transfer genes is present in the same cell (1, 11). One class of nonconjugal but mobilizable plasmids encodes plasmid-specific protein(s) which interacts at its own specific *oriT* site(s) to initiate DNA processing for transfer. These plasmids utilize the transfer apparatus established by a coreident transfer factor.

A general model for the processing of DNA to initiate transfer, based primarily on experiments with the F factor of *Escherichia coli*, includes the formation of a relaxation complex at *oriT_F* and subsequent site- and strand-specific nicking at this site (26). Following this initiation reaction, the nicked single strand is transferred to the recipient cell in a 5'-to-3' direction and the complementary strand is synthesized in the new host. The untransferred DNA strand serves as a template for replacement synthesis in the donor cell, although this event is not required for successful DNA transfer. For detailed reviews of conjugative transfer involving F and selected R factors, see Willetts and Skurray (25) and Guiney and Lanka (3).

A great deal of attention has been directed to the study of transfer among the broad-host-range transfer factors of the IncP family. This group of transfer factors is capable of

promoting conjugation within the family *Enterobacteriaceae* as well as to diverse gram-negative organisms, including the obligate anaerobe *Bacteroides fragilis*, the plant pathogens of the *Rhizobium* family, and the tumor-inducing *Agrobacterium* spp. (3). Furthermore, the IncP plasmids can supply some of the essential *trans*-acting transfer functions to a wide variety of plasmids that contain specific *oriT* sites and as a result promote mobilization of these nonconjugal plasmids *in trans* (3). The biochemical processes involved in the initiation of DNA transfer in the IncP plasmids have already been established (1, 3, 16).

Hecht et al. have previously described (6, 7) the isolation of the 9.6-kb transposon Tn4399, which does not contain any known antibiotic resistance determinant, from *B. fragilis*. Acquisition of Tn4399 by nonconjugal plasmids allowed these plasmids to be transferred from *B. fragilis* donor cells to *B. fragilis* and *E. coli* recipients (6). Therefore, it was concluded that Tn4399 was a conjugal transposon. We show here that Tn4399-containing plasmids can be mobilized in *E. coli* by IncP α and IncP β plasmids. All Tn4399 functions required for the mobilization process are contained within a 2.8-kb region internal to the transposon. Thus, in *E. coli*, Tn4399 is a mobilizing transposon. We have identified two proteins of 36.4 and 16.4 kDa and an *oriT* region that are required for the mobilization process in *E. coli*.

MATERIALS AND METHODS

Bacterial strains and media. Some important characteristics of the bacterial strains and plasmids used in these experiments are described in Table 1. *E. coli* strains were grown in ML broth for liquid culture (14) and selected on Luria or Mueller-Hinton (Difco Co., Detroit, Mich.) broth solidified with 1.5% agar and supplemented with antibiotics as appropriate. The following antibiotic concentrations (micrograms per milliliter) were used for selective media unless otherwise specified: ampicillin (Amp), 200; chloramphenicol

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant phenotype or genotype	Comment	Source and/or reference(s)
Bacterial strains			
<i>E. coli</i>			
BMH71-18	<i>mutS</i>		Promega; 10
DH5 α	<i>recA</i>		21
DW1030	Sp ^r <i>recA</i>		19
HB101	Sm ^r <i>recA</i>		21
JM109	<i>recA1</i>		Promega; 21
RVNal/ λ	Nal ^r Rec ⁺	λ -resistant derivative of RVNal	12
SM10(λ pir)	Kan ^r	RP4 integrated into chromosome	K. Berger; 23
Plasmids			
Transfer factors			
pRK231	Kan ^r Tet ^r Tra ⁺ ^a	IncP α plasmid	2, 13
R751	Tp ^r Tra ⁺	IncP β plasmid	3
Cloning vectors			
pAlter	Tet ^r	Plasmid vector used for selection of oligonucleotide-directed mutants	Promega
pBR322	Amp ^r Tet ^r	<i>E. coli</i> cloning vector derived from ColE1	21
pUC7/pUC19	Amp ^r	<i>E. coli</i> cloning vector derived from ColE1 via several steps	21
Constructs containing Tn4399 or derivatives			
pAlter-2.8	Tet ^r	pAlter + 2.8-kb <i>NdeI-HindIII</i> fragment of Tn4399	This study
pCGM01 Δ 1	Amp ^r	pDWH22 with deletion of 3.8 kb from the left end of Tn4399	This study
pCGM03	Amp ^r Kan ^r	pDWH22 + Tn1000CK insertion	This study
pCGM03 Δ 1	Amp ^r	pDWH22 with deletion of 5.0 kb from the left end of Tn4399	This study
pCGM300	Amp ^r	pUC19 + Tn4399	This study
pCGM328	Amp ^r	pUC7 + 2.8-kb <i>NdeI-HindIII</i> fragment of Tn4399	This study
pCGM338	Amp ^r	pUC7 + 3.8-kb <i>NdeI-NdeI</i> fragment of Tn4399	This study
pCGM341-353	Amp ^r Kan ^r	pCGM328 + Tn1000CK insertions	This study
pCGM361-372, pCGM390	Amp ^r	pUC7 + subcloned fragments of Tn4399	This study
pCGM400	Amp ^r	pBR322 + Tn4399	This study
pCGM410	Amp ^r	pBR322 + 1.0-kb <i>HindIII-NdeI</i> fragment of Tn4399	This study
pCGM428	Amp ^r	pBR322 + 2.8-kb <i>NdeI-HindIII</i> fragment of Tn4399	This study
pCGM438	Amp ^r	pBR322 + 3.8-kb <i>NdeI</i> fragment of Tn4399	This study
pCGM501	Cm ^r Kan ^r	pDWH10 + Kan ^r insertion	This study
pCGM528	Cm ^r	pACYC184 + 2.8-kb <i>NdeI-HindIII</i> fragment of Tn4399	This study
pCGM530	Cm ^r	pCGM528 + <i>BspHI</i> fill-in, mutant <i>orf4</i>	This study
pCGM601	Tet ^r	<i>orf4</i> mutant derivative of pAlter-2.8	This study
pDWH10	Cm ^r	pACYC184 + Tn4399	6
pDWH22	Amp ^r Tet ^r	pGAT400 Δ BglII::Tn4399	6

^a Tra⁺, capable of self-transfer.

(Cm), 25; kanamycin (Kan), 25; nalidixic acid (Nal), 50; spectinomycin (Sp), 50; streptomycin (Sm), 50; trimethoprim (Tp), 10; and tetracycline (Tet), 10. Antibiotics were obtained from Sigma Chemical Co., St. Louis, Mo. Indicator media contained 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) (Boehringer Mannheim, Indianapolis, Ind.) at 40 μ g/ml.

Plasmid mobilization experiments. Plasmid mobilization experiments from *E. coli* donor cells to *E. coli* recipients were conducted with the protocols of Furste et al. (1), with minor modifications. A mid-log-phase donor culture (4.5 ml) and a recipient culture (0.5 ml) were mixed and attached to the surface of a Nalgene filter (Nalge Company, Rochester, N.Y.) by vacuum filtration. The filters were transferred to the surface of Luria agar plates and incubated aerobically overnight at 37°C. The filters were placed in 5 ml of ML

broth, and the cells were resuspended by vigorous agitation. Transconjugants were enumerated by plating on selective media. The mobilization frequency for each plasmid is reported as the mean $\pm$ the standard error of the number of colonies containing the antibiotic resistance marker(s) of the plasmid divided by the number of transconjugants that acquired the IncP transfer plasmid in the same experiment.

Mobilization in trans. To confirm that plasmids were being mobilized in *trans* rather than as a result of the formation of cointegrates with the mobilizing plasmids followed by resolution, plasmid DNA was transformed into a donor strain, SM10(λ pir) (23), which carries the transfer genes of IncP α plasmid RP4 integrated into the chromosome. Because RP4 DNA is immobilized in the *E. coli* chromosome, it can supply transfer functions in *trans* to mobilizable plasmids without transferring itself to the recipient strain. SM10(λ pir)

donor strains containing experimental plasmids were mated with a suitable recipient strain, RVNal/ λ , by the mobilization protocol described above. Transconjugants were screened for the absence of cointegrate formation by isolation of the plasmid DNA and subsequent analysis using multiple restriction endonucleases.

Complementation experiments. Complementation experiments between mutant and wild-type *mocA* and *mocB* genes were conducted as described above, except that the donor strain contained three plasmids: the *mocA* or *mocB* mutant plasmid, the respective wild-type genes cloned into a compatible plasmid, and the IncP mobilizing plasmid. Similarly, to localize the minimal *cis*-required *oriT* region, each donor strain contained three compatible plasmids: a plasmid containing the subcloned fragment of the "mobilization cassette," the wild-type mobilization cassette, and the IncP mobilizing plasmid. Therefore, essential mobilization functions encoded by the mobilization cassette were provided by the wild-type copy in *trans* to the subcloned fragments being tested. Mobilization of each of the three plasmids present in the donor strain was scored independently by selection of the plasmid-specific antibiotic resistance determinant(s) encoded by the respective plasmids.

Recombinant DNA techniques. Plasmid DNA isolation and restriction endonuclease analysis were done as described previously (21). All restriction endonucleases, Klenow DNA polymerase, and T4 DNA polymerase were purchased from New England Biolabs (Beverly, Mass.) and used according to the supplier's directions with the following exceptions. The restriction enzymes *Mae*II and *Esp*I were purchased from Boehringer Mannheim and United States Biochemical (Cleveland, Ohio), respectively. S1 nuclease was purchased from Promega (Madison, Wis.).

DNA sequencing. The Sequenase method (version 2.0; United States Biochemical) was used according to the supplier's directions to determine nucleotide sequences of Tn4399. The oligonucleotide primers from the ends of Tn1000 were used as previously described (24). The sequence of the ends of the mobilization cassette was determined after cloning the 2.8-kb region into pGAT400 Δ BglII and using a synthetic primer corresponding to nucleotides 1022 to 1042 of pBFTM10 (5). Additional primers were synthesized to sequence the remaining gaps of the 2.8-kb region on both strands, (see Fig. 2).

Construction of plasmids pCGM400 and pCGM300. pDWH22, a transposition product of Tn4399 insertion into pGAT400 Δ BglII (6), was the source of Tn4399 for further plasmid constructions. The plasmid was restricted with *Bgl*II and *Eco*RI restriction endonucleases to generate a 10.6-kb fragment containing the intact Tn4399. Following separation on a 0.8% agarose gel and purification using the GeneClean II kit from Bio 101 (La Jolla, Calif.), this fragment was ligated into pBR322 restricted with *Bam*HI and *Eco*RI. The ligation mixture was used to transform competent *E. coli* HB101, and pCGM400 was identified as pBR322 containing the Tn4399 insertion.

To construct pCGM300, the polylinker site of pUC19 was first altered by cloning the synthetic oligonucleotide 5'-GCT AGATCTAGCTGCA3' into the *Pst*I site of the existing polylinker of pUC19. Introduction of this oligonucleotide into pUC19 results in a new *Bgl*II site flanked by *Pst*I sites in the pUC19 polylinker. The 10.6-kb *Bgl*II-*Eco*RI restriction fragment containing Tn4399, purified as described above, was ligated into the modified pUC19 vector restricted with *Bgl*II and *Eco*RI. The ligation mixture was used to transform

competent HB101 cells, and pCGM300 (a Tn4399-containing pUC plasmid) was isolated.

pDWH10, a third Tn4399-containing plasmid used in these studies, is described by Hecht and Malamy elsewhere (6).

Tn1000CK mutagenesis. Plasmid pOX38 carrying Tn1000CK, a derivative of Tn1000 encoding resistance to cefoxitin (Cef) and kanamycin (unpublished data), was used to create Tn1000CK insertion mutations in pDWH22 and pCGM328 (a pUC7 derivative containing a 2.8-kb restriction fragment from Tn4399) as previously described (24).

Mutagenesis of *orf5*. A kanamycin resistance determinant from Tn903 was isolated from pRI133 (9) by restriction of this plasmid with *Bam*HI. The 1.2-kb fragment containing the Kan^r cartridge was purified from a 0.8% agarose gel using the GeneClean II kit from Bio 101. After the ends of this cartridge were filled in by using Klenow polymerase, it was ligated into the unique *Bst*XI site (located in the N terminus of *orf5*) of pDWH10 (Tn4399-containing pACYC184) to create pCGM501. The ligation mix was used to transform competent *E. coli* HB101 cells, and transformants were selected on Luria agar plates containing chloramphenicol, kanamycin, and streptomycin.

Mutagenesis of *orf4*, *orf8*, and *orf9*. pCGM530 was created by introducing a frameshift mutation at the unique *Bsp*HI site of pCGM528. Purified plasmid DNA was treated with the restriction endonuclease *Bsp*HI, the resulting 3' overhang was filled in by using Klenow polymerase, and the blunt-ended DNA was ligated. This type of mutation results in a 4-bp frameshift in *orf4*, *orf8*, and *orf9*. The ligation mixture was used to transform competent HB101 cells, and resultant transformants were screened for the loss of the *Bsp*HI site.

An additional mutation in this region was created by using the Altered Sites in vitro Mutagenesis System (Promega) to introduce a site-directed base pair substitution, G $\rightarrow$ A at position 1216. According to the supplier's directions, the 2.8-kb region of interest was cloned into the supplied phagemid, pAlter, and single-stranded DNA was isolated by using the supplied helper phage R408. A mutagenic oligonucleotide containing the single base substitution was annealed to the single-stranded template in the presence of an ampicillin repair oligonucleotide and extended with T4 DNA polymerase. The inactivated ampicillin resistance gene of the pAlter phagemid is repaired by the repair oligonucleotide at the same time that the desired mutation is introduced by the mutagenic oligonucleotide. Ampicillin-resistant candidates were selected by 125- μ g/ml ampicillin and 15- μ g/ml tetracycline selection. The mutation into *orf4* was confirmed by sequence analysis. Introduction of this mutation into the mobilization cassette sequence does not alter the primary coding sequence of *orf9* but results in premature termination of the primary sequence encoded by *orf4* in the opposite orientation. Consequently, the potential 143-amino-acid protein encoded by *orf4* is terminated after only 58 amino acids by a UGA codon.

Subclones. Various-sized fragments of Tn4399, which were generated by reaction with restriction endonucleases, were cloned into the polylinker site of an intermediate cloning vector, pUC7 or pUC19, after treatment with Klenow DNA polymerase to fill in the ends of the insert DNA. To create pCGM390 (pUC7 containing *orf8* and *orf9* corresponding to nucleotides 1097 to 1538 of the 2.8-kb sequence), the insert DNA was treated with S1 nuclease prior to treatment with Klenow DNA polymerase and then ligated to the pUC7 vector. DNA ligation mixtures were used to transform competent DH5 α cells to Amp^r on X-Gal indicator

TABLE 2. Mobilization of nonconjugal plasmids by IncP plasmids in the presence (+) and absence (–) of Tn4399 in *cis*^a

Plasmid	Presence of Tn4399 in <i>cis</i>	Mean frequency of mobilization ($\pm$ SE) with ^b :	
		pRK231	R751
pBR322	–	$<10^{-7}$	$<10^{-7}$
pCGM400	+	$1.9 (\pm 0.9) \times 10^{-5}$	$3.6 (\pm 1.2) \times 10^{-6}$
pUC7	–	$<10^{-7}$	$<10^{-7}$
pCGM300	+	$1.3 (\pm 0.8) \times 10^{-4}$	$3.6 (\pm 0.3) \times 10^{-5}$
pACYC184	–	$<10^{-7}$	$<10^{-7}$
pDWH10	+	$1.9 (\pm 0.6) \times 10^{-5}$	$4.2 (\pm 1.5) \times 10^{-7}$

^a Plasmids were mobilized from DW1030 containing pRK231 or R751 to HB101.

^b Mobilization frequency is defined as in the text: number of plasmid transconjugants/number of IncP transconjugants in the same experiment. Mean mobilization frequencies with no IncP plasmid were $<10^{-7}$.

media. Each subclone was independently assayed for mobilization in the presence of IncP plasmids pRK231 and R751.

Nucleotide sequence accession number. The double-stranded DNA sequence of the 2.8-kb region of Tn4399 including the mobilization cassette has been submitted to GenBank and given the accession number L20975.

RESULTS

Mobilization of Tn4399-containing plasmids by IncP plasmids in *E. coli*. Hecht and Marmy have shown previously that the transposition of Tn4399 from the chromosome of a *B. fragilis* strain to a nonconjugal chimeric plasmid such as pGAT400ΔBglII, allows the transfer of the plasmid from *B. fragilis* donor cells to *Bacteroides* and *E. coli* recipient cells (6).

In order to study the properties of Tn4399 in *E. coli* in the absence of all other transfer sites and genes (such as the *ori*_{RP4} region of pDG5 [4], a component of pGAT400ΔBglII), Tn4399 was cloned into the ColE1 derivatives pBR322 and pUC19 and the p15A derivative pACYC184. pBR322, pUC19, and pACYC184 are nonconjugal plasmids that lack self-transfer sites and functions and are not mobilized by IncP plasmids RP4 and R751. The presence of Tn4399 did not result in self-transfer of pCGM400 (pBR322 containing Tn4399), pCGM300 (pUC19 containing Tn4399), or pDWH10 (pACYC184 containing Tn4399) from *E. coli* HB101 to *E. coli* recipients. Thus, the presence of Tn4399 does not confer transfer ability to *E. coli* nonconjugal plasmids in *E. coli*. However, plasmids pCGM400, pCGM300, and pDWH10 could be mobilized in the presence of IncP plasmids pRK231 (an RP4 derivative) or R751. As shown in Table 2, all three plasmids were mobilized by either IncP plasmid. pCGM400 was mobilized by either pRK231 or R751 at frequencies of 10^{-5} to 10^{-6} , while pCGM300 was mobilized at frequencies of 10^{-4} to 10^{-5} compared with the transfer of the IncP plasmids. Tn4399-containing plasmids isolated from the recipient cells were unaltered by the mobilization event (data not shown). pDWH10 was also mobilized without alteration by both IncP plasmids at frequencies of 10^{-5} to 10^{-7} compared with the transfer of the IncP plasmids. Neither pCGM400, pCGM300, nor pDWH10 could be mobilized at a detectable frequency in the absence of a coresident IncP plasmid. These results indicate that there is a strict requirement for a transfer factor in the same *E. coli* cell for mobilization of Tn4399-containing plasmids.

TABLE 3. Localizing the mobilization region to a 2.8-kb restriction fragment of Tn4399^a

Plasmid ^b	Mean frequency of mobilization ($\pm$ SE) with ^c :	
	pRK231	R751
pCGM01Δ1	^d	$2.9 (\pm 0.5) \times 10^{-6}$
pCGM03Δ1	*	$<10^{-7}$
pCGM438	$4.2 (\pm 0.7) \times 10^{-4}$	$6.6 (\pm 0.6) \times 10^{-6}$
pCGM428	$8.7 (\pm 2.0) \times 10^{-4}$	$7.8 (\pm 2.0) \times 10^{-6}$
pCGM410	$<10^{-7}$	$<10^{-7}$
pCGM338	$1.1 (\pm 0.4) \times 10^{-2}$	$1.3 (\pm 0.2) \times 10^{-4}$
pCGM328	$6.4 (\pm 1.5) \times 10^{-3}$	$3.7 (\pm 1.1) \times 10^{-4}$

^a Plasmids were mobilized from DW1030 containing pRK231 or R751 to HB101.

^b Refer to Fig. 1 for structure of plasmids.

^c Mobilization frequency is defined as in the text: number of plasmid transconjugants/number of IncP transconjugants in the same experiment. Mean mobilization frequencies with no IncP plasmid were $<10^{-7}$.

^d *, these plasmids were not assayed for mobilization in the presence of pRK231, since they contain *ori*_{RP4}.

To confirm that there is no physical interaction between the experimental plasmids and the IncP plasmids, a series of control experiments was conducted as described in Materials and Methods. pCGM300 and negative-control plasmid pUC7 were each transformed into a mobilizing donor strain, SM10(λpir), which contains plasmid RP4 immobilized in the *E. coli* chromosome (23). Transformants were mated with the recipient strain RVNal/λ. Plasmid pCGM300 was mobilized from this background at the same frequency as seen when mobilizing plasmid pRK231 was present in the donor strain in *trans*. DNA isolated from transconjugants was unaltered (data not shown). This result indicates that physical interaction between the IncP mobilizing plasmid pRK231 and the Tn4399-containing plasmid pCGM300 is not required for mobilization. Mobilization of pUC7, a plasmid lacking any known transfer genes or sites, could not be detected from the SM10(λpir) donor strain (mobilization frequency, $<10^{-9}$).

Defining an essential mobilization function by deletion analysis. A series of overlapping deletions from the left end of Tn4399 was generated by restriction digestion of pDWH22 (one of the original pGAT400ΔBglII::Tn4399 plasmids) and

FIG. 1. Schematic representation of Tn4399 and derivatives. (A) Tn4399. IR, 13 of 14 bp that form a perfect inverted repeat at the ends of Tn4399 (7); *, 5 extra bp at right end of Tn4399 (7). The hatched box represents the sequence presented in Fig. 2. (B) Fragments of the mobilization region contained in pCGM01Δ1, pCGM03Δ1, pCGM438, pCGM338, pCGM528, pCGM428, pCGM328, and pCGM410.

then assayed for mobilization in the presence of R751. As shown in Table 3 and Fig. 1, pCGM01Δ1, which lacks 3.8 kb from the left end of Tn4399, was mobilized by R751 at a frequency of $2.9 (\pm 0.5) \times 10^{-6}$, approximately the same frequency as that of the entire Tn4399 cloned into pBR322. This result indicates that the left end of Tn4399 was not required for the mobilization process in *E. coli*. More extensive deletions removing 5 kb or more from the left end of Tn4399, as in pCGM03Δ1, become mobilization deficient; thus, the boundary of an essential mobilization function is located between the left ends of deletions pCGM01Δ1 and pCGM03Δ1.

Subcloning the mobilization region of Tn4399. When a 3.8-kb *NdeI* restriction fragment from the center of Tn4399 was cloned into pBR322 to generate pCGM438 or into pUC7 (a pUC19 plasmid with a different polylinker cloning site) to generate pCGM338 (Fig. 1) and placed in *trans* to pRK231 or R751, the plasmids were mobilized at greater than or equal to the frequency seen for the intact Tn4399 (Table 3). As before, there were no detectable alterations to the transferred plasmids. This result indicates that neither the left nor the right end of Tn4399 is required for mobilization in *E. coli*. The 3.8-kb *NdeI* restriction fragment could be further dissected with *HindIII* to generate two fragments of 2.8 and 1.0 kb shown schematically in Fig. 1, which were independently cloned into pBR322 or pUC7 and assayed for mobilization in the presence and absence of the IncP plasmids. As shown in Table 3, pCGM428 (pBR322 containing the 2.8-kb *NdeI-HindIII* fragment of Tn4399) or pCGM328 (pUC7 containing the same 2.8-kb fragment) was efficiently mobilized by the IncP plasmids at a frequency equal to or above that seen for the intact Tn4399. pCGM328 was also tested for mobilization from the SM10(λpir) mobilizing strain as described for pCGM300 and was shown to be mobilized at high frequency and without alteration from this background. Thus, no physical interaction between pCGM328 and the IncP plasmids is required for mobilization.

As shown in Table 3, pCGM410 (pBR322 containing the 1.0-kb *HindIII-NdeI* fragment of Tn4399) could not be mobilized by the IncP plasmids above a low background level. At low frequency ($<1 \times 10^{-9}$), some transfer of this plasmid was detected on the selective plates. Analysis of these rare transconjugants revealed extensive alterations of the original plasmid DNA (data not shown). Several other subclones from Tn4399 which did not contain the 2.8-kb restriction fragment bounded by *NdeI* and *HindIII* sites could not be mobilized by the IncP plasmids. We have designated the region required for mobilization that is contained within the 2.8-kb *NdeI-HindIII* restriction fragment a mobilization cassette.

DNA and protein sequence analysis of the mobilization cassette. As will be described in more detail below, a series of insertions of Tn1000CK into the 2.8-kb fragment was generated. The complete DNA sequence of the 2.8-kb *NdeI-HindIII* fragment was determined by using the sites of Tn1000CK insertions and oligonucleotide primers specific for the ends of the Tn1000CK to determine the adjacent sequence (Fig. 2). Additional oligonucleotides complementary to the determined sequences were synthesized to fill in gaps in the sequence and to complete sequencing of the complementary strand. The locations of the Tn1000CK insertions were determined by DNA sequence analysis, and the wild-type sequences at these insertion sites were confirmed independently.

Analysis of the DNA sequence revealed the presence of several potential open reading frames (ORFs) on both

strands of the sequence capable of encoding proteins ranging in size from 8 to 36.4 kDa. As shown in Fig. 3B, the top strand could code for six proteins. *orf1*, *orf2*, *orf3*, and *orf4* code for potential products of 150, 106, 115, and 143 amino acids, respectively, based on a methionine start codon for each of the potential proteins. *orf5*, a slightly larger ORF, codes for a product of 318 amino acids. *orf6* codes for a potential gene product of 129 amino acids. In the opposite orientation, three ORFs, *orf7*, *orf8*, and *orf9*, encoding respective potential products of 96, 106, and 69 amino acids could be identified. Because there are no methionines in *orf8*, the potential gene product of 106 amino acids is the length of the entire ORF. To determine which of the ORFs were involved in mobilization, we created insertion and deletion mutations in this region.

The gene product of *orf5*, MocaA, functions in *trans* and is required for mobilization. Insertional mutagenesis of pCGM328 containing the mobilization cassette was performed with Tn1000CK to disrupt potential coding sequences. Individual insertion mutants, pCGM341 through 353, which interrupt *orf1*, *orf2*, *orf3*, *orf5*, *orf6*, and *orf7* were assayed for their ability to be mobilized in the presence of the IncP plasmids. As shown schematically in Fig. 3 and quantitatively in Table 4, the plasmids containing the Tn1000CK insertions in *orf5* could not be mobilized. While two of these insertions, pCGM349 and pCGM350, also disrupt the potential coding sequence of *orf7*, insertions pCGM351 and pCGM352 which lie outside of the coding sequence for *orf7* but disrupt *orf5* are still mobilization deficient. An additional mutation in *orf5* but not in *orf7* was created by cloning a *Kan^r* determinant into the N terminus of *orf5* to form pCGM501. In the presence of either IncP plasmid, pCGM501 was also mobilization deficient. These results suggest that the 36.4-kDa protein product encoded by *orf5* is an essential mobilization factor. Therefore, we have designated this gene *mocA* (for mobilization cassette) and its corresponding gene product MocaA.

In the presence of an intact Tn4399 on a compatible plasmid in *trans*, the *mocA* mutant plasmid was mobilized at wild-type frequencies. For these experiments the donor cell contained three compatible plasmids: a *mocA* mutant plasmid, the Tn4399-containing plasmid pDWH10, and the transfer factor pRK231 or R751. As shown in Table 4, all *mocA* mutant plasmids were mobilized at high frequency in the presence of pRK231 or R751 when Tn4399 was provided in *trans*. This result establishes that a product of the *orf5* region, presumably the 36.4-kDa MocaA protein, is a *trans*-acting factor in the mobilization process.

Insertions into *orf1*, *orf2*, or *orf6* did not affect mobilization frequency, suggesting that these ORFs do not code for essential mobilization functions. Though the insertions into *orf7*, as present in pCGM349 and pCGM350, did lead to a mobilization-deficient phenotype, *orf5* was also disrupted by these insertions. Therefore, the role of *orf7*, if any, has yet to be determined. Two insertions into *orf3* were isolated: one, pCGM343, had no altered phenotype, and the second, pCGM344, showed slightly reduced mobilization by pRK231 and R751 (data not shown). Further studies will determine whether this ORF has an auxiliary though not essential role in the mobilization process.

MocaA shares homology with DNA relaxases. The derived protein sequence of MocaA was compared with that of other plasmid mobilization proteins and found to share similarities with DNA-relaxing enzymes encoded by a wide variety of conjugal or mobilizable plasmids (Fig. 4). These include agrobacterial Ti and Ri plasmids, a mobilizable plasmid from

FIG. 3. (A) Sites of mutations in the mobilization cassette and their respective mobilization phenotypes. Inverted triangles, *Tn1000CK* insertions in the mobilization cassette; x, site of a site-directed base pair change which introduces a translation termination signal in *orf4* but which remains sense in *orf9*, pCGM601; y, site of a *Bsp*HI fill-in, pCGM530; z, site of a *Kan*^r insertion, pCGM501; +, mobilization frequency in the range 10⁻³ to 10⁻⁴; -, mobilization-deficient phenotype with mobilization frequency <10⁻⁷; ↓, reduced frequency of transfer (10⁻⁴ to 10⁻⁵). (B) Summary of ORFs in the 2.8-kb region including the mobilization cassette.

Thiobacillus ferrooxidans, broad-host-range IncPα and IncPβ plasmids, and mobilizable plasmids from *Staphylococcus aureus*. Alignment of representative mobilization proteins according to the scheme of Pansegrau and Lanka (15) reveals a 14-amino-acid sequence motif which is highly conserved among DNA relaxases.

MocA of Tn4399 can also be aligned with a more generalized version of the relaxase motif (not shown), designated motifs 2a and 2 by Ilyina and Koonin (8), which highlights the conserved histidine residues embedded in a highly hydrophobic sequence in both Rep and Mob classes of rolling circle replication proteins.

The gene product of *orf4*, MocB, is required for mobilization and functions in *trans*. The DNA region located between nucleotides 1040 and 1507 (Fig. 2) contains three potential ORFs: *orf4* in the top strand codes for a 16.4-kDa protein, and in the bottom strand *orf8* codes for an 11.7-kDa protein and *orf9* codes for a 7.9-kDa protein. Insertion of 4 bp into the unique *Bsp*HI site in this region, as in pCGM530, disrupts all three ORFs. As shown in Table 5, the plasmid containing the insertion, pCGM530, could not be mobilized

even though the donor cells contained the IncP mobilizer plasmid in *trans* to this plasmid, suggesting that the DNA sequence disrupted by this insertion encodes a critical mobilization factor. When the wild-type mobilization cassette was provided in *trans* on a compatible plasmid, pCGM328, the mutant plasmid pCGM530 could be mobilized at high frequency by pRK231 or R751. pCGM366 containing a 541-bp *Xmn*I-*Bst*XI restriction fragment of the mobilization cassette including the intact *orf4*, *orf8*, and *orf9* can also complement insertion mutant pCGM530 in the presence of the IncP plasmids, demonstrating that the protein(s) encoded by this region could be supplied in *trans*. Therefore, one of these ORFs, *orf4*, *orf8*, or *orf9*, provides an essential function for the mobilization process. Cloned restriction fragments containing *orf8* but not *orf4* or *orf9* could not complement insertion mutants in this region (data not shown).

Evidence that *orf8* is not essential for mobilization was provided by pCGM03, a *Tn1000CK* insertion mutation into pDWH22 (pGAT400Δ*Bgl*II::Tn4399) at site 1451. This insertion lies in the middle of *orf8* and at the extreme C terminus of *orf4*. In the presence of R751, pCGM03 could be mobilized at wild-type frequencies (data not shown), indicating that *orf8* and the last 6 amino acids of *orf4* are not essential for mobilization by R751 in *E. coli*.

Further complementation analysis showed that a subclone containing an intact *orf8* and *orf9* but with a 19-amino-acid N-terminal deletion in *orf4*, pCGM390, could complement pCGM530 to slightly reduced mobilization frequencies in the

TABLE 4. Mobilization of pCGM328 derivatives with *Tn1000CK* insertions in *orf5* by IncP plasmids in the presence (+) and absence (-) of Tn4399 in *trans*^a

Plasmid	Presence of Tn4399	Mean frequency of mobilization (±SE) with ^b :	
		pRK231	R751
pCGM349	-	<10 ⁻⁷	<10 ⁻⁷
pCGM349	+	4.8 (±1.5) × 10 ⁻⁵	2.8 (±0.8) × 10 ⁻⁶
pCGM350	-	<10 ⁻⁷	<10 ⁻⁷
pCGM350	+	3.8 (±1.1) × 10 ⁻⁵	4.6 (±1.4) × 10 ⁻⁶
pCGM351	-	<10 ⁻⁷	<10 ⁻⁷
pCGM351	+	1.6 (±0.6) × 10 ⁻⁵	3.4 (±1.3) × 10 ⁻⁶
pCGM352	-	<10 ⁻⁷	<10 ⁻⁷
pCGM352	+	1.9 (±0.7) × 10 ⁻⁵	1.1 (±0.4) × 10 ⁻⁶

^a Plasmids were mobilized from DW1030 containing pRK231 or R751 to HB101. Refer to Fig. 3 for sites of these *TN1000CK* insertions in *orf5*.

^b Mobilization frequency is defined as in the text: number of plasmid transconjugants/number of IncP transconjugants in the same experiment. Mean mobilization frequencies with no IncP plasmid were <10⁻⁷.

MocA (Tn4399)	102	Y	I	V	A	R	H	T	D	R	E	H	P	H	C	H	I	V	F	N	R	V	D	N	D
MobA (pC221)	94	V	A	V	Y	T	H	T	D	K	D	H	Y	H	N	H	I	V	I	N	S	V	D	L	E
Rix1 (pS194)	94	V	A	V	Y	T	H	T	D	K	D	H	Y	H	N	H	I	V	I	N	S	V	N	L	E
NikB (R64)	153	Y	V	S	A	V	H	T	D	T	D	N	L	H	V	V	A	V	N	R	V	H	P	E	
VirD2 (pTIA6)	126	Y	L	T	A	Y	H	V	D	R	D	H	P	H	L	H	V	V	N	R	R	E	L	L	
VirD2 (pRIA4)	126	Y	L	T	A	F	H	I	D	R	D	H	P	H	L	H	V	V	N	R	R	E	L	L	
Tral (RP4)	104	R	V	S	A	V	H	H	T	D	T	D	N	L	H	I	H	I	A	I	N	K	I	B	P
Tral (R751)	104	R	I	S	A	V	H	N	D	T	D	N	L	H	I	H	I	A	I	N	K	I	B	P	
MobA (pTF-FC2)	51	A	I	Y	G	L	H	A	D	T	D	N	L	H	L	H	L	A	I	N	R	V	H	P	E
Rix (pC223)	94	V	A	V	Y	T	H	N	D	T	D	H	V	H	N	H	I	V	I	N	S	I	D	L	E

FIG. 4. Alignment of DNA relaxases including MocA of the mobilization cassette of Tn4399 according to the scheme of Pansegrau and Lanka (15). The boxed sequence represents the common motif among DNA relaxases. The sequence position of the first amino acid residue for each plasmid is indicated by the number to the right of the plasmid name.

TABLE 5. Mobilization of pCGM530, a derivative of pCGM528 with an insertion in the *orf4-orf9* region, by IncP plasmids in the presence and absence of subcloned fragments of this region in *trans*^a

Plasmid	Plasmid in <i>trans</i>	Mean frequency of mobilization ($\pm$ SE) with ^b :	
		pRK231	R751
pCGM528		$7.1 (\pm 0.9) \times 10^{-5}$	$5.6 (\pm 1.2) \times 10^{-6}$
pCGM530		$<10^{-7}$	$<10^{-7}$
pCGM530	pCGM328	$2.2 (\pm 0.6) \times 10^{-4}$	$1.8 (\pm 0.5) \times 10^{-5}$
pCGM530	pCGM366	$2.0 (\pm 0.3) \times 10^{-5}$	$5.3 (\pm 2.1) \times 10^{-6}$

^a Plasmids were mobilized from DW1030 containing pRK231 or R751 to HB101. The insertion site in pCGM530 is designated y in Fig. 3.

^b Mobilization frequency is defined as in the text: number of plasmid transconjugants/number of IncP transconjugants in the same experiment. Mean mobilization frequencies with no IncP plasmid were $<10^{-7}$.

presence of pRK231 and R751 (data not shown). In this case, the truncated *orf4* could code for a potential restart protein beginning at position 1163, leading to a product of 102 amino acids.

To determine which ORF, *orf4* or *orf9*, was required for mobilization, the Altered Sites in vitro Mutagenesis System was used to introduce a site-directed base pair substitution in this region as described in Materials and Methods. The resultant mutant, pCGM601, which retains the primary coding sequence of *orf9* but terminates after 58 amino acids of *orf4* was assayed for mobilization in the presence of the IncP β plasmid R751. pCGM601 could not be mobilized in the presence of R751 (Table 6). To confirm that the *trans*-acting protein encoded by this region had been mutated by the single base pair substitution, the mobilization assay was repeated in the presence of an intact copy of the mobilization cassette supplied on a compatible plasmid, pCGM528. Mobilization was restored to the wild-type frequency (Table 6), indicating that the essential protein could be supplied in *trans*. Since ORF4 was the only protein disrupted by this mutation, we conclude that *orf4* encodes the essential *trans*-acting protein required for mobilization. Therefore, we have designated this gene *mocB* and the corresponding gene product MocB. The potential role of *orf9*, if any, has yet to be determined.

No significant homology or similarity between MocB and transfer proteins of pRK231 (TraI, TraJ, TraK, TraL, TraM, TraG, and TraH) or R751 (TraI, TraJ, and TraK) could be detected by the Bestfit or FASTA programs of the GCG DNA analysis software.

Localization of the *oriT* region within the mobilization cassette. While mutations of *mocA* and *mocB* can be com-

TABLE 6. Mobilization by R751 of pCGM601, a derivative of pAlter-2.8 containing a nonsense mutation in *orf4*, in the presence (+) and absence (–) of pCGM528^a

Plasmid	Presence of pCGM528	Mean frequency of mobilization ($\pm$ SE) ^b
pAlter	–	$<10^{-7}$
pAlter-2.8	–	$1.7 (\pm 0.7) \times 10^{-6}$
pCGM601	–	$<10^{-7}$
pCGM601	+	$9.6 (\pm 1.0) \times 10^{-6}$

^a Plasmids were mobilized from DW1030 containing pRK231 or R751 to HB101. The location of the mutation in pCGM601 is designated x in Fig. 3.

^b Mobilization frequency is defined as in the text: number of plasmid transconjugants/number of IncP transconjugants in the same experiment. Mean mobilization frequencies with no IncP plasmid were $<10^{-7}$.

plemented in *trans*, some deletion mutations within the mobilization cassette could not be mobilized by either IncP plasmid, even in the presence of MocA and MocB provided by a compatible plasmid. Thus, a *cis*-acting *oriT* site involved in DNA transfer must be located in the region mapped by these nonmobilizable deletion derivatives of the mobilization cassette.

To localize the minimal *cis*-required region of the mobilization cassette, the *oriT*, subcloned fragments were assayed for mobilization in *trans* to the essential MocA and MocB functions and the IncP transfer factor in a Rec[–] donor cell. As shown schematically in Fig. 5, small restriction fragments of the mobilization cassette were cloned into pUC7 to create plasmids pCGM361 through 372. These plasmids were introduced by transformation into the Rec[–] *E. coli* HB101 strain, also containing pDWH10 and either pRK231 or R751. The resulting transformant containing three plasmids was mated to a suitable recipient, and the transfer of the pCGM361-372 plasmids was measured.

With this strategy, the *oriT* region of the mobilization cassette was localized to a 199-bp restriction fragment (Fig. 2 and 5). Plasmid pCGM370 containing pUC7 and the 199-bp restriction fragment of the mobilization cassette was mobilized at a frequency of $1.3 (\pm 0.2) \times 10^{-3}$ and $2.6 (\pm 0.7) \times 10^{-5}$ compared with the transfer of the IncP plasmids pRK231 and R751, respectively. DNA isolated from transconjugants revealed that there were no alterations in the plasmid DNA compared with DNA isolated from the donor cells (data not shown). Inspection of the DNA sequence of this fragment reveals the presence of two inverted repeats and the N-terminal 49 amino acids of MocB.

DISCUSSION

Transposon Tn4399 was originally identified on the basis of its ability to restore the transfer properties of deletion derivatives of a shuttle plasmid containing pBFTM10 in *B. fragilis*. We then asked if the transfer functions of Tn4399 could be expressed in *E. coli*. Our initial experiments established that Tn4399 could not confer transfer ability to non-conjugal plasmids in *E. coli*. It is possible that the requirements for transfer in *B. fragilis* differ from those in *E. coli*, and that Tn4399 does not encode all of the necessary functions to promote formation of mating aggregates as well as to initiate DNA transfer in *E. coli*. However, in the presence of the IncP plasmids pRK231 or R751 in *E. coli*, mobilization of Tn4399-containing plasmids is observed. The consistently higher frequency of mobilization by pRK231 [and by SM10(λ pir) for representative plasmids tested] may suggest that IncP α plasmids are more active than IncP β plasmids, or perhaps IncP α plasmids can interact more efficiently with Tn4399 sequences for mobilization.

When assayed for mobilization in *trans* to IncP α and IncP β plasmids pRK231 and R751 in *E. coli*, pCGM400 (pBR322 containing Tn4399) and pDWH10 (pACYC184 containing Tn4399) were efficiently mobilized to *E. coli* recipient strains compared with control vectors without Tn4399 insertions (background mobilization frequencies). The ability of Tn4399-containing plasmids to be mobilized by pRK231 and R751 suggests that at a minimum Tn4399 must possess an *oriT* sequence at which conjugal DNA processing can be initiated. The lower efficiency of mobilization of Tn4399-containing plasmids in *E. coli* compared with that of the IncP plasmids themselves may result from poor expression of the *mocA* and *mocB* genes in *E. coli*. Differences in mobilization frequencies among vectors containing the same Tn4399

FIG. 5. (A) Schematic representation of the mobilization cassette which was dissected to localize the *oriT* region. (B) Fragments of the mobilization region contained in pCGM361 through 372. The size of each subcloned fragment is indicated (in base pairs) above each fragment. +, mobilization frequency in the range 10^{-3} to 10^{-4} ; -, mobilization-deficient phenotype with mobilization frequency $<10^{-7}$.

sequences (such as pCGM300 and pDWH10 [Table 2]) could result from the differences in copy number among plasmid vectors, i.e., from differing levels of MocA and MocB. By deletion analysis, the boundary of an essential mobilization function was localized to between the left ends of pCGM01Δ1 and pCGM03Δ1. By assaying various subclones of Tn4399, we determined that a 2.8-kb region internal to the transposon was sufficient to confer mobilization potential to nonconjugal plasmids in *E. coli*. These results establish that the left and right ends of Tn4399 are not involved in mobilization in *E. coli*, and therefore, mobilization of Tn4399-containing plasmids is likely to be independent of Tn4399 transposition. The higher mobilization frequency of mobilization cassette-containing plasmids compared with that of Tn4399-containing plasmids remains unexplained.

The mobilization cassette isolated from Tn4399 is the first such mobilization region characterized from a conjugal transposon. As a conjugal transposon, Tn4399 in *B. fragilis* is capable of disseminating genetic information by both transposition and conjugation (6). Unlike other conjugal transposons such as Tn916 and Tn1545, in which transfer proceeds via a circularized intermediate of the excised transposon (22), the Tn4399 mobilization cassette promotes transfer in the absence of the ends of the transposon. Furthermore, the 13 of 14 bp that form a perfect inverted repeat and the five extra bases at the right end of Tn4399 identified by sequencing the ends of the transposon (7) must not play a role in the mobilization process in *E. coli*.

Analysis of the mobilization frequencies of Tn1000CK insertions throughout the mobilization region revealed that plasmids containing disruptions in the fifth ORF, which we have designated *mocA*, could not be mobilized by either IncP plasmid. *mocA* encodes a potential 36.4-kDa protein which can be supplied in *trans* by other Tn4399-carrying plasmids. Because mutations in *mocA* could not be complemented by the TraH, TraI, TraJ and TraK DNA-processing proteins of pRK231 or R751, *mocA* seems to be Tn4399

specific. Indeed, MocA shares a common 14-amino-acid sequence motif with nine other confirmed and potential DNA relaxases encoded by a wide variety of conjugal or mobilizable plasmids from both gram-negative and gram-positive hosts (15). Confirmed DNA relaxases interact specifically with their respective target regions to create a nicked open circular form of plasmid DNA prior to transfer. In the case of RP4, the TraI relaxase catalyzes a site-specific cleaving-joining reaction of single-stranded DNA (16). The relationship of the relaxases suggests that the genes and mechanism of transfer in such diverse organisms may have been derived from a common ancestor.

The genetic organization of the mobilization cassette isolated from transposable element Tn4399 is quite similar to other two-component plasmid mobilization systems, including those of pBFTM10 from *B. fragilis* and pC221 from *S. aureus*, as well as the multicomponent mobilization system of pTF-FC2 of *T. ferrooxidans* (20). For these plasmids it seems that a MocA-type product processes the DNA at the *oriT* site to initiate the transfer process. One or more transfer functions required to establish and regulate effective mating contacts between donor and recipient bacteria are supplied in *trans* by a coresident mobilizing factor with relatively little specificity for the plasmid being mobilized. The role of the host chromosome in such mobilization experiments has yet to be determined.

The protein and DNA sequence of the Tn4399 mobilization cassette has also been compared with the transfer region of pBFTM10, a self-transmissible plasmid of *B. fragilis* that can also be mobilized in *E. coli* by the IncPβ plasmid R751 (5). Two pBFTM10 genes, *btgA* and *btgB*, encoding proteins of 23.2 and 33.8 kDa, respectively, are required for the mobilization process in *E. coli*. Preliminary studies with deletion derivatives of pBFTM10 have localized the *oriT* site of pBFTM10 directly upstream of *btgA* and *btgB* (6a). Though the organization of the mobilization regions of these two plasmids may be similar, we can detect no significant

similarity between the *moc* genes and the *btg* genes or between the gene products of Tn4399 and pBFTM10.

The *oriT*₄₃₉₉ region within the mobilization cassette was localized to a 199-bp fragment, bounded by the restriction sites *Xmn*I and *Mae*II. On the basis of DNA sequence analysis, this minimal *cis*-required region could not code for an intact protein. This sequence does not show homology to the IncP-type nick site consensus sequence. Analysis of the *oriT*₄₃₉₉ region, including the formation of relaxasomes, will determine whether single-stranded nicking occurs within this 199-bp fragment. If so, the nick site of Tn4399 will be analyzed for homology to other nick sites that interact with IncP plasmids.

A 46-bp interval within the 199-bp *oriT* sequence of Tn4399 shares 64% identity to the *oriT* region of pC221 (bp 3111 to 3172) of *S. aureus* (18). pC221 encodes two mobilization genes, *mobA* and *mobB*, which are sufficient to initiate relaxosome formation at the *oriT* nick site of pC221 (17). *MobA* of pC221 and *MocA* of Tn4399 share the DNA-relaxase motif (15) (Fig. 4), although there is no significant similarity between *MobB* of pC221 and *MocB* of Tn4399.

In *B. fragilis*, Tn4399-containing chimeric plasmids are capable of transferring to *B. fragilis* and *E. coli* recipients in the absence of any other known plasmid or transfer factor. Studies are in progress to determine whether the same *oriT*₄₃₉₉ defined in *E. coli* and the *MocA* and *MocB* mobilization proteins are used for transfer from *B. fragilis* donor cells.

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