***Erwinia uredovora* 20D3 Contains an IncM Plasmid**

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Erwinia uredovora was lysogenized with phages Mucts62 and D108-1, a mini derivative of D108cts10 carrying a Cm^r marker. The 20D3(Mucts62)(D108-1) lysogen was induced and used as donor in a mating with a phage-resistant *Escherichia coli*; the Cm^r marker was transferred to this recipient strain at low frequency. The analysis of the Cm^r transconjugants indicated that they had acquired the pULG3 plasmid of *E. uredovora* associated with a D108-1 prophage. Subsequent transfer of these pULG3 derivatives within *E. coli* indicated that pULG3 was conjugative. Incompatibility experiments showed that pULG3 is a member of the IncM group.

INTRODUCTION

Erwinia uredovora belongs to the *herbicola* group of the genus *Erwinia*. These are yellow pigmented enterobacteriaceae, often isolated as epiphytes. The peculiarity of the *uredovora* species resides in their ability to destroy the uredia and urediospores of the rust fungus *Puccinia* (Buchanan & Gibbons, 1974).

Recent work shows that *E. uredovora* 20D3: (i) produces a phage tail-like bacteriocin active against the phytopathogenic strains *Erwinia carotovora* and *Erwinia amylovora* (Thiry-Braipson *et al.*, 1982); (ii) contains three plasmids with molecular sizes of 260 kb (pULG1), 216 kb (pULG2) and 88 kb (pULG3), with pULG1 involved in both yellow pigmented and thiamin-independent phenotypes (Thiry, 1984); and (iii) adsorbs and propagates the phages Mu and D108 (Faelen *et al.*, 1981). Mu and D108 are mutator phages that are heteroimmune and closely related. The analysis of heteroduplexes between the DNA of the two phages indicates an homology of 95% between their genomes (Hull *et al.*, 1978). Both phages are efficient transposable elements (for a review, see Toussaint & Résibois, 1983). Mini-Mu and mini-D108 derivatives that have lost their lytic functions but conserved the ability to transpose were isolated (Faelen *et al.*, 1978; Résibois *et al.*, 1981). The frequency of transposition of mini-phages that only provide the *A* gene product is low but is strongly stimulated in the presence of a helper phage that can be either Mu or D108 (A. Toussaint, personal communication). Since the mini-phage and its helper each code for a thermolabile repressor, their transposition can be induced very efficiently at 42 °C and repressed at low temperature. Upon induction of a lysogen containing a mini *A*⁺ phage and a helper, both genomes are packaged and the lysate can be used to introduce the mini-phage into sensitive bacteria.

This paper reports the use of Mu and D108-1, a mini-D108 derivative carrying Tn9, to demonstrate that one of the plasmids of *E. uredovora* 20D3 is conjugative. The procedure involved: (i) the isolation of a lysogen carrying D108-1 and Mucts62 helper phage; (ii) the induction of the prophages resulting in the transposition of Mucts62 and D108-1 to random locations in the genome of 20D3; and (iii) selection for the conjugative transfer of the Cm^r marker of D108-1 by mating the induced lysogen with an appropriate recipient.

METHODS

Bacteria, phages and plasmids. These are listed in Table 1. All plasmids are maintained in *Escherichia coli* C600 Rif.

Media. Bacteria and phages were grown in L broth (Miller, 1972). Mu and D108 lysates were titrated on L agar (L broth containing 1.2% w/v, agar). Minimal medium was M9 (Miller, 1972) with 0.2% (w/v) glucose. The Lac phenotype was tested on MacConkey agar supplemented with 1% (w/v) lactose. Antibiotics were used at 20 µg ml⁻¹ for ampicillin (Ap), tetracycline (Tc), chloramphenicol (Cm) and kanamycin (Km), 50 µg ml⁻¹ for streptomycin (Sm) and rifampicin (Rif), and 100 µg ml⁻¹ for spectinomycin (Sp). The sulphonamide (Su), neomycin (Nm) and trimethoprim (Tp) markers were tested with sensitivity discs obtained from l'Institut Pasteur, Paris, France. Phages were diluted in SM buffer (Tris/HCl, 10⁻² M; NaCl, 10⁻¹ M; MgSO₄, 2 × 10⁻² M, pH 7.4) and bacteria in MgSO₄, 10⁻² M.

Preparation of phage lysates. Phage lysates were prepared by heat-induction of *E. coli* lysogens using the procedure of Bukhari & Ljungquist (1977).

Lysogenization of 20D3 by Mucts62. A drop of Mucts62 lysate at ~10⁹ p.f.u. ml⁻¹ was spotted on a lawn of 20D3 on L agar and incubated overnight at 32 °C. Bacteria from the lysis area were streaked on L agar. Isolated colonies were tested for the presence of Mu prophage by stabbing into a lawn of sensitive bacteria on L agar. Lysogenic bacteria produced phages which lysed the indicator lawn.

Mating. Spot matings were done as described by Miller (1972).

Preparation and analysis of plasmid DNA. Large-scale DNA preparations were made according to Currier & Nester (1976). Two types of small-scale extractions were used. The first was the mini-preparation of Kado & Liu

Table 1. *Bacterial strains, phages and plasmids*

The symbols of bacterial genes are according to Bachmann (1983) and of plasmid markers according to Novick *et al.* (1976).

Strain	Characters	Source or reference
<i>E. ureidovor</i>		
20D3	Prototroph	ATCC 19321, received from C. Kado, Davis, USA
20D3(Mucts62)	As 20D3 with (Mucts62)	This work
20D3(Mucts62)(D108-1)	As 20D3 with (Mucts62)(D108-1)	This work
<i>E. coli</i>		
JM260	$\Delta(ara-leu) his argE lac gal mtl xyl$ $recB sbcB rpsL supE hsr hsm^+$	F. Sanchez, UNAM, Mexico
JMM260	As JM260 but Mu ^r	This work
CSH23	$\Delta(pro-lac) supE thi rpsE$	Miller (1972)
MXR	$\Delta(pro-lac) galE thi recA$	Faelen <i>et al.</i> (1978)
C600	$thr leu thi lacY tonA supE$	Appleyard (1954)
C600 Rif	Spontaneous Rif ^r mutant of C600	P. Pohl, INRV, Belgium
Phage	Characters	Reference
Mucts62	Thermoinducible	Howe (1973)
D108cts10	Thermoinducible	Hull <i>et al.</i> (1978)
DM1	Recombinant between D108cts10 and Mu; has D108 immunity and Mu host range	Toussaint <i>et al.</i> (1983)
D108-1	D108cts10 $\Delta(B-S)$ carrying a Tn9 in the remaining part of the <i>S</i> gene	Résibois <i>et al.</i> (1981)
Plasmid	Inc group	Marker
RIP185	B	Km Tp
R16a	C	Km Ap Su
Flac	FI	Lac
pIP24	FII	Tc
R124drd2	FIV	Tc
pIP523	H1	Tc
RIP112	I α	Km Nm
TP114	I2	Km
R721	I δ	Sm Tp
R69-2	M	Km Ap
RP4	P	Km Ap Tc
R135	M	Tc
<div style="display: flex; align-items: center;"> <div style="margin-right: 10px;"> </div> <div> </div> </div>		
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(1981). The second was derived from the alkaline extraction procedure of Birnboim & Doly (1979): an overnight culture in L broth (20 ml) was centrifuged at 7000 *g* for 5 min at 4 °C. The pellet was resuspended in 3 ml of a lysis solution (2 mg lysozyme ml⁻¹, 50 mM-glucose, 10 mM-EDTA, 25 mM-Tris/HCl, pH 8.0) and placed on ice for 30 min. A 1% (w/v) SDS solution (6 ml) in 0.2 M-NaOH was added. The tube was gently inverted, left for 5 min at 0 °C and 4.5 ml of a renaturing solution (3 M-sodium acetate, pH 4.8) added. The tube was then placed on ice for 1 h. High molecular weight DNA and protein-SDS complexes were removed by centrifugation at 17000 *g* for 5 min at 4 °C. Cloudy material that remained in the supernatant was removed by a second centrifugation at 20000 *g* for 5 min at 4 °C. The plasmid DNA in the supernatant was precipitated with two volumes of cold ethanol for 30 min at -20 °C, pelleted by centrifugation at 6000 *g* for 5 min at 4 °C, and resuspended in 2 ml of a solution containing 0.1 M-sodium acetate and 50 mM-Tris/HCl (pH 8.0). After a second ethanol precipitation and centrifugation, the pellet was briefly dried in a vacuum desiccator, dissolved in 0.5 ml pancreatic RNAase (0.1 mg ml⁻¹) in 5 mM-Tris/HCl (pH 7.5) and incubated for 30 min at 37 °C. Plasmid DNA could be restricted at this step, although better results were obtained after a third ethanol precipitation. The DNA pellet was finally resuspended in 250 µl TE buffer (10 mM-Tris/HCl, 1 mM-EDTA, pH 7.8).

Electrophoresis of plasmid DNA was carried out in 0.7% (w/v) agarose horizontal slab gels (140 × 130 × 3 mm) in Tris/acetate buffer (McDonell *et al.*, 1977) at 100 V for 6 h or, when the DNA was restricted, at 25 V for 16 h. Gels were stained with ethidium bromide (0.5 µg ml⁻¹) for 45 min. The DNA was visualized and photographed on a UV transilluminator type C-63 (Lab Center, Belgium). Negatives were scanned with a 3CS Joyce-Loebl photomicroscope (Van Oortmerssem, Antwerpen, Belgium) and the relative amounts of DNA in the various restriction fragments were determined by measuring the area under the peaks. Restriction enzymes were obtained from Boehringer and used according to the instructions supplied.

Incompatibility tests. Tra⁺ plasmids were transferred into appropriate strains. The unstable inheritance of two plasmids in the lineage of at least four different clones which possessed both molecules was tested as described by Datta (1979).

RESULTS AND DISCUSSION

Isolation of 20D3(Mucts62)(D108-1) lysogens

The genomes of Mu, D108 and DM1 (a hybrid between D108cts10 and Mucts62; Toussaint *et al.*, 1983) contain an invertible DNA segment, the G region, that carries genes specifying the host range of the phages (van de Putte *et al.*, 1980; Gill *et al.*, 1981). *Erwinia uredovora* 20D3 is sensitive to these three phages which have their host range specified by the G loop in the '—' orientation (Faellen *et al.*, 1981). Phage lysates that contained both G(—) and G(+) particles were prepared by heat induction of *E. coli* (Mucts62) or (DM1)(D108-1) lysogens and titrated on *E. coli* JM260 and *E. uredovora* 20D3. Both lysates gave 10 to 300 fewer plaques on 20D3 than on JM260 (see Table 2). The difference in the number of p.f.u. detected with the same lysate between these two hosts not only reflected a difference in the number of G(—) and G(+) particles in the lysates but also an incomplete protection of the phage DNA against the host-specific restriction system of *E. uredovora* (Toussaint & Schoonejans, 1982). 20D3(Mucts62) lysogens were isolated as described in Methods. One of the lysogens was purified, infected with a DM1/D108-1 lysate and plated on L agar supplemented with Cm. Cm^r colonies resulting from

Table 2. *Titration of Mucts62 and DM1/D108-1 lysates on E. coli JM260 and E. uredovora 20D3*

Phage lysates were prepared by heat-induction of *E. coli* lysogens, as described in Methods, and the number of transductants was determined by spotting dilutions of the lysate on lawns of the sensitive bacteria plated on L agar supplemented with chloramphenicol.

Lysate	Indicator strain	No. of p.f.u. ml ⁻¹	No. of Cm ^r transductants ml ⁻¹
Mucts62	JM260	3 × 10 ⁹	—
	20D3	10 ⁷	—
DM1/D108-1	JM260	2.5 × 10 ⁹	2.5 × 10 ⁶
	20D3	1 × 10 ⁸	1.5 × 10 ⁵
	20D3(Mucts62)	3 × 10 ⁸	1 × 10 ⁵

—, Not applicable.

Fig. 1. Plasmids of *E. uredoovora* 20D3 (extreme left and right) and of six different *E. coli* CSH23(pULG3::D108-1) strains. The DNA was extracted by the procedure of Kado & Liu (1981).

the integration of mini-D108-1 appeared at a frequency of 10^{-3} p.f.u. $^{-1}$. The presence of the D108-1 prophage in the Cm^r transductants was confirmed by showing that these strains had D108 immunity and produced only Muets62 infectious particles. All transductants tested retained the plasmids of the parental strain (data not shown).

Plasmid-mediated transfer of D108-1 to E. coli

20D3(Muets62)(D108-1) was grown in L broth at 32 °C to a density of 2×10^8 bacteria ml $^{-1}$ and heated for 20 min at 42 °C to induce the transposition of D108-1. The transferability of the plasmids labelled by D108-1 was determined by looking for the transfer of the Cm^r marker to a plasmid-free *E. coli* recipient strain. The Sm^r JMM260 strain was chosen as recipient because of its *hsr* genotype (which prevents restriction of unmodified incoming DNA) and its resistance to Mu (which prevents transduction of Cm^r by the D108-1 genomes packaged in Mu capsids). Cm^r Sm^r transconjugants were isolated at a frequency of about 10^{-7} per donor cell and 20 of these were purified and tested for the transferability of their Cm^r traits to *E. coli* strain CSH23 at 32 °C (this temperature was used to repress the D108 function). All clones were able to donate the Cm^r marker at frequencies ranging from 10^{-9} to 2×10^{-5} per donor cell.

Plasmid content of the E. coli Cm^r transconjugants

The plasmids of *E. uredoovora* 20D3 and six *E. coli* CSH23 Cm^r transconjugants were extracted by the method of Kado & Liu (1981) and their electrophoretic mobilities were compared (Fig. 1). 20D3 contained three plasmids with molecular sizes of 260 kb (pULG1), 216 kb (pULG2) and 88 kb (pULG3). The CSH23 Cm^r transconjugants each carried one plasmid that migrated differently in each clone and was smaller than pULG1 and pULG2. Four molecules have mobilities close to the one expected for a pULG3::D108-1 plasmid. Homology between pULG3 and these plasmids was investigated by comparing the *Eco*RI digestion patterns of the DNA extracted by alkaline lysis (Birnboim & Doly, 1979) from 20D3 and CSH23 Cm^r clones. This extraction method resulted in the isolation of pULG3 largely devoid of the other plasmid DNA from strain *E. uredoovora* 20D3. A similar result was obtained using the method of Currier &

Fig. 2. Restriction of pULG3 with *Eco*RI and scanning of the pattern. The plasmids were purified according to Currier & Nester (1976). λ DNA digested with *Eco*RI was used as a size standard. The areas under peaks 3, 7 and 8 were found to be twice the expected values. Therefore, each of these peaks must correspond to two digestion fragments that have similar electrophoretic mobilities.

Nester (1976), if no care was taken during the shearing and denaturation/renaturation steps. The *Eco*RI digest of pULG3 isolated from 20D3 is composed of 11 bands (Fig. 2). The microdensitometry analysis indicated that bands 3, 7 and 8 correspond to doublets. pULG3 thus contained 14 sites for *Eco*RI. The molecular size of pULG3 as deduced from the restriction fragments was 86.1 ± 2.5 kb. The *Eco*RI restriction patterns of pULG3 and the plasmids carrying the Cm^r marker (Fig. 3) show that 10 electrophoretic bands were common to pULG3 and each of the Cm^r plasmids. This clearly identified pULG3 as a conjugative plasmid. The different Cm^r plasmids showed the same additional fragment of 3 kb that corresponds to a 3 kb *Eco*RI fragment present in D108-1 DNA. Moreover, the replacement of one of the *Eco*RI fragments of pULG3 by two 'junction' fragments was detected in some of the DNA preparations. The different Cm^r plasmids, as deduced from their *Eco*RI patterns, ranged between 80 and 100 kb in size. A molecular size of 96 kb could be expected for pULG3 with a single precise insertion of D108-1. The heterogeneity in the size of the pULG3::D108-1 plasmids is not surprising, however, since it was previously reported that transposition of Mu from one replicon to another may result in rearrangements (insertion or deletion) of the recipient replicon (Chaconas *et al.*, 1981; Coelbo *et al.*, 1980; Maynard-Smith *et al.*, 1980). The transposition of D108-1 to random locations in pULG3, as well as the rearrangements induced by the mini-phage (Toussaint *et al.*, 1981), could be related to the differences in transfer frequency of the various pULG3::D108-1 plasmids.

Determination of the incompatibility group of pULG3

The incompatibility group of pULG3 was investigated using pULG3-8, one of the pULG3::D108-1 plasmids that transferred most efficiently. The molecular size of pULG3-8 was estimated to be 96 kb. Members of IncB, IncC, IncFI, IncFII, IncFIV, IncHI1, IncI α , IncI2, IncI δ , IncM or IncP incompatibility groups (see Table 1) were introduced in JMM260(pULG3-8) and the segregation of the markers of both plasmids was investigated. pULG3-8 could stably coexist with all the plasmids tested except R69-2, which belongs to the IncM group. Member-

Fig. 3. *Eco*RI restriction of different pULG3::D108-1 plasmids compared with *Eco*RI restriction of pULG3 (track 1). The plasmids were prepared by a method derived from that of Birnboim & Doly (1979), as described in Methods. The arrow indicates the characteristic internal *Eco*RI fragment of D108-1.

Table 3. *Incompatibility between IncM plasmids and pULG3-8*

Clones were tested as described in Methods. The markers used for the selection of the transfer and to detect segregation of the plasmids were Km^r for R69-2, Tc^r for R135 and Cm^r for pULG3-8. Symbols * and † denote the resident and incoming plasmid, respectively.

Strain	No. of clones tested	No. of clones which lost the marker of:		
		The resident plasmid (*)	The incoming plasmid (†)	Both plasmids
C600(R69-2)*(R135)†	180	151	8	1
C600(R69-2)	20	0	—	—
C600(R135)	20	—	0	—
JMM260(pULG3-8)*(R69-2)†	60	27	8	0
JMM260(pULG3-8)	20	0	—	—
JMM260(R69-2)	20	—	0	—
C600(R69-2)*(pULG3-8)†	60	5	13	0
C600(R69-2)	20	0	—	—
C600(pULG3-8)	20	—	0	—
CSH23(pULG3-8)*(R135)†	115	11	23	0
CSH23(pULG3-8)	20	0	—	—
CSH23(R135)	20	—	0	—

ship of pULG3 in the IncM family was further supported by the introduction of pULG3-8 into C600(R69-2) or the transfer of R135, another IncM plasmid, into CSH23(pULG3-8) (Table 3). R69-2 and R135 were also transferred to *E. uredo* 20D3. Both plasmids were unstable in this host and were lost (in the absence of selection pressure) at a frequency of 50% to 95%. Attempts to isolate 20D3 strains that maintained R69-2 or R135 stably and were cured of pULG3 were

unsuccessful. The difference in the behaviour of these plasmids in *E. coli* and *E. uredovora* is under investigation.

The IncM plasmids form a large group in the enterobacteriaceae (Hedges *et al.*, 1973; Richards & Datta, 1979). These plasmids have molecular sizes of 65 to 105 kb and were mostly isolated from the genera *Proteus*, *Klebsiella* and *Serratia* (Jacob *et al.*, 1977). Most of them carry transposable elements which determine resistance to antibiotics (Chabbert *et al.*, 1979).

Transfer of any of the pULG3::D108-1 plasmids from *E. uredovora* to *E. coli* did not provide the recipient with any detectable function other than resistance to chloramphenicol. This suggested that pULG3 did not code for the bacteriocin produced by *E. uredovora* 20D3. Recent results indicate that the genetic determinants of this bacteriocin are located in the host chromosome (M. Thiry & G. Thiry, unpublished results). Experiments are in progress to determine whether the transfer system of pULG3 can mobilize the chromosome of *E. uredovora*.

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