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Transfer, Maintenance and Expression of P Plasmids in Strains of Cowpea Rhizobia

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Seven slow-growing strains of cowpea rhizobia (*Rhizobium* sp.) were tested for ability to receive the P plasmids pRD1 and R68.45 from *Escherichia coli*. The frequency of transfer was 2×10^{-4}–2×10^{-6} for five strains including CB756, but no transfer was detected for the other two. The *his–nif* segment of pRD1 was lost at high frequency in CB756 transconjugants whilst pRD1 and R68.45 *tra* and antibiotic resistance genes were stable through 50–100 generations in non-selective media even though growth rates of CB756 were almost halved in transconjugant clones. Furthermore, plasmids were maintained in three strains tested during passage through root nodules, although there were some differences in symbiotic expression between transconjugants and their wild-type parents. A colour change was effected by CB756 in the presence of 6-cyanopurine and therefore could possibly be used as an indicator of *nif* expression in addition to acetylene reduction in culture.

INTRODUCTION

Rhizobia belonging to the 'cowpea miscellany' are of special significance to nitrogen fixation research because they usually form nodules on a broad spectrum of tropical legumes, in contrast to other *Rhizobium* spp. which effectively nodulate only one or a few legume species. Hence, the genetic and biochemical factors leading to promiscuity of nodulation in cowpea rhizobia may be significant for long-term endeavours to extend the host range of root-nodule bacteria. In addition, several cowpea strains express high nitrogenase activities in defined culture, a desirable feature for the study of *nif* gene function in *Rhizobium* which avoids other determinants necessary for effective symbiosis.

Despite the advantages offered for the study of *Rhizobium nif* genes by the slow-growing cowpea strains, relatively little is known about the ease with which *nif* mutants might be isolated or the extent to which useful plasmids can be transferred to and from these strains. Glutamine auxotrophs with impaired nitrogenase expression have been isolated from the cowpea strain 32H1 (Ludwig & Signer, 1977) and *nif* mutants have been reported for *R. japonicum* (Maier & Brill, 1976), also a slow-growing species but more strongly host-specific than cowpea rhizobia. Recently, *R. japonicum* was shown to receive only certain P plasmids and then at very low frequency (Kuykendall, 1979).

The aim of the work presented here was to study the transfer, maintenance and expression of P plasmids in strains of cowpea rhizobia, and to develop methods which would aid the isolation of Nif⁻ mutants. Strain CB756 was chosen for particular study because, although regarded as a slow-grower, it (i) grew relatively fast in a modified LNB5 medium (doubling time 3–4 h); (ii) gave consistently higher rates of acetylene reduction in culture compared

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with, for example, strain 32H1; and (iii) was found to produce a bacteriocin active against other strains of cowpea rhizobia (B. Dreyfus, A. H. Gibson & E. A. Schwinghamer, unpublished results). Bacteriocin production is a useful genetic character and a property associated with symbiotic genes on a plasmid in *R. leguminosarum* strain 248 (Johnston *et al.*, 1978 *a*).

METHODS

Organisms. The strain numbers and origins of the bacteria used are shown in Table 1. The *Rhizobium* sp. strain CB756 resembles strain 32H1 in serological and symbiotic characteristics but differs in colony morphology and origin.

Media and growth conditions. Yeast mannitol (YM) medium contained, per litre: K_2HPO_4, 0·5 g; $MgSO_4$, 0·1 g; $CaCl_2$, 0·04 g; $FeCl_3$, 0·004 g; sodium glutamate, 0·5 g; mannitol, 5·0 g; yeast extract (bakers' yeast infusion), 50 ml. Modified LNB5 (modLNB5) medium (from Maier *et al.*, 1978) contained, per litre: $NaH_2PO_4.H_2O$, 0·15 g; $CaCl_2.2H_2O$, 0·15 g; $MgSO_4.7H_2O$, 0·25 g; FeEDTA, 25 mg; $MnSO_4.H_2O$, 10 mg; $CuSO_4.5H_2O$, 0·04 mg; $CoCl_2.6H_2O$, 0·025 mg; KI, 0·78 mg; inositol, 0·1 g; thiamin.HCl, 0·01 g; nicotinic acid, 1 mg; sodium gluconate, 0·5 g; sodium glutamate, 0·5 g; yeast extract, 0·1 g; the pH was adjusted to 5·5 with concentrated HCl before autoclaving. NF12–CS7 medium (Pagan *et al.*, 1975) was optimized for nitrogenase activity in strain 32H1 as described by Gibson *et al.* (1976). Seedling nutrient agar contained, per litre: K_2HPO_4, 0·05 g; KH_2PO_4, 0·05 g; $Ca_3(PO_4)_2$, 2·0 g; $FePO_4$, 0·5 g; $CaCO_3$, 0·1 g; $MgSO_4$, 0·2 g; NaCl, 0·1 g; $FeCl_3$, 0·01 g; KCl, 0·2 g; Mn, Zn, Cu and Mo, trace amounts; agar (Difco), 15 g.

Media for growing *Klebsiella pneumoniae* and *Escherichia coli* were nutrient broth (NB) and nutrient agar (NA) (Oxoid nutrient agar no. 2), Davis & Mingioli (1950) minimal medium (DM), and NFDM (NH_4^+-free medium) (Cannon *et al.*, 1974).

Liquid cultures of *Rhizobium* strains were inoculated with fresh growth taken from YM or modLNB5 agar slopes or plates. Flasks contained 5–10% of their volume as liquid, and were shaken in air at 29 °C.

Nitrogenase activity. The soft agar overlay method of Pankhurst & Craig (1978) was employed using 25 ml Universal bottles containing 8 ml of NF12 or modLNB5 + sodium succinate (12·5 mM) solid media..

Table 1. *Organisms used*

Rhizobium sp.	Origin	Source
CB756	*Macrotyloma africanum* (Wilczek) Verdc.; Zimbabwe	R. A. Date, CSIRO, Brisbane, Australia
CB756(Spc^r)	Spontaneous mutant of CB756 resistant to spectinomycin at 200 μg ml^{-1}	—
32H1	'Nitragin' strain	J. C. Burton, Nitragin Co., Milwaukee, U.S.A.
61B9	'Nitragin' strain	J. C. Burton
QA922	*Neonotonia wightii* (Arn.) Lackey; Brisbane, Australia	A. Diatloff, D.P.I., Brisbane, Australia
CB1015	*Vigna radiata* (L.) Wilczek; Haringhata, Bangladesh	R. A. Date
CB1024	*Macrotyloma uniflorum* (Lam.) Verdc.; Coimbatore, India	R. A. Date

Escherichia coli	Relevant characteristics of plasmid		Source and reference
SB1801(pRD1)	IncP1	Km Tc Cb His$^+$ Nif$^+$	ARC UNF collection (Dixon *et al.*, 1976)
1230(R68.45)	IncP1	Km Tc Cb	A. W. B. Johnston, John Innes Institute, Norwich, U.K. (Haas & Holloway, 1976)

Klebsiella pneumoniae	Relevant phenotype	Source and reference
CK263	His$^-$ NifA$^-$ Hsd$^-$	ARC UNF collection (Dixon *et al.*, 1977)

Plasmid transfer. (i) From *E. coli* to *Rhizobium* sp. *Escherichia coli* donors and cowpea strain recipients were grown to the late exponential phase in NB and in modLNB5 medium, respectively. Drops of each culture were applied together on the surface of an agar plate containing a mixture of modLNB5 and NA (4:1) (after Beringer, 1974) and incubated at 32 °C for 3–5 d after which the cells were washed and plated for selection of transconjugants. To aid selection of plasmid-bearing *Rhizobium* sp. transconjugants, use was made of the natural resistance of these strains to chloramphenicol and nalidixic acid at levels very much higher than those to which *E. coli* is resistant. Usually chloramphenicol at 50 μg ml^{-1} and nalidixic acid at 30 μg ml^{-1} were incorporated in modLNB5 or YM plates along with kanamycin (for QA922, 100 μg ml^{-1}; CB756 and 61B9, 150 μg ml^{-1}; CB1015 and CB1024, 400 μg ml^{-1}) and ampicillin (for CB756 and CB1024, 25 μg ml^{-1}; 61B9 and CB1015, 200 μg ml^{-1}; QA922, 400 μg ml^{-1}). Additionally, these strains, with the exception of CB1015, are resistant to rifampicin at approximately 100 μg ml^{-1} and, including CB1015, to gentamicin (> 200 μg ml^{-1}) and trimethoprim (> 400 μg ml^{-1}); these characteristics could also be used to counterselect plasmid donor strains.

(ii) From *Rhizobium* sp. to *K. pneumoniae.* Drops of liquid cultures were applied to mixed media plates as in (i) and incubated at 29 °C for 3–5 d. Transconjugants were selected on NA containing antibiotics, on DM minimal medium for *his* transfer, or on NFDM for selection of *nif* gene transfer. *Rhizobium* sp. strains used in this study fail to grow on these media.

Test for symbiotic properties of transconjugants. Symbiotic nitrogen fixation was assessed by growing transconjugants in association with Siratro [*Macroptilium atropurpureum* (DC.) Urb.]. The plants were cultured under bacteriologically controlled conditions, with the roots growing on a slope of the nitrogen-free seedling nutrient agar within a test tube and the shoots exposed to the atmosphere (Gibson, 1963). Pre-germinated seeds were sown in the tubes, inoculated 3 d later with 10 d cultures of single colony isolates of the transconjugants grown on YM agar, and provided with sterile water as necessary. The plants were grown for 28 d in a louvre-shaded glasshouse at a mean day length of 14 h. Temperatures in the first experiment were 24 °C (day) and 16 °C (night) but these appeared too low for optimum plant growth. For the second experiment, therefore, temperatures were raised to 29 °C (day) and 21 °C (night). At harvest, the roots were examined for the presence of nodules and the shoots were cut off, oven-dried at 80 °C and weighed. The differences between the weights of inoculated and uninoculated plants were regarded as an index of nitrogen fixation.

RESULTS

Growth and nitrogenase activity of strains CB756 and 32H1

Growth rates of strains CB756 and 32H1 were measured in both YM and modLNB5 media. Strain CB756 had a generation time of 3 h in modLNB5 medium contrasted to 16 h in YM medium; strain 32H1 grew with 4 and 12 h doubling times in modLNB5 and YM media, respectively. Thus modLNB5 medium was used throughout this work to obtain relatively fast, exponential growth of cells in liquid culture, although it offered no advantage as a solid growth medium.

Optimal conditions for acetylene reduction with strain CB756 were obtained using the soft agar overlay method with NF12 medium. Strain CB756 consistently showed 20–50 % higher activities than did 32H1 typically attaining 70 nmol ethylene formed per hour per culture 8 d after inoculation. High activities were also obtained using modLNB5 medium supplemented with 12·5 mM-sodium succinate, but in this case activity had diminished by 12–14 d after inoculation compared with 22–24 d using NF12 medium.

Colour formation from 6-cyanopurine

6-Cyanopurine was shown to be a colour indicator for *nifA* gene expression in *K. pneumoniae* (MacNeil & Brill, 1978). Conditions were defined under which cultures of CB756 turned dark pink in the presence of 6-cyanopurine and which were similar to those for optimum acetylene reducing activity. 6-Cyanopurine at 5 mM was contained in 8 ml of NF12 1·8 % agar in 25 ml bottles. Approximately 5 × 10^7 cells (0·05 ml of a late-exponential phase culture) were suspended in 0·18 ml semi-soft (1%) NF12 agar and layered on top of the 8 ml NF12 agar base. The bottles were loosely capped and incubated at 29 °C in air. After 8 d the layer of cells turned pink and then darkened over the next few days. No colour was observed if the volume of the semi-soft layer was more than 0·25 ml or if the concentration of

Table 2. *Frequency of plasmid transfer to strains of Rhizobium sp.*

| | Plasmid transfer frequency* | |
| | E. coli donor | |
Rhizobium sp. recipient	SB1801(pRD1)	1230(R68.45)
CB756	1×10^{-5}	1×10^{-4}
CB756(Spc^r)	5×10^{-6}	$6 \cdot 3 \times 10^{-5}$
CB1024	2×10^{-4}	2×10^{-5}
QA922	4×10^{-6}	2×10^{-6}
61B9	$<1 \times 10^{-8}$	$<1 \times 10^{-8}$
CB1015 .	$<1 \times 10^{-8}$	$<1 \times 10^{-8}$
32H1	$1 \cdot 1 \times 10^{-5}$	Not tested

* Selection was for kanamycin and ampicillin resistance.

6-cyanopurine was decreased to 1 mM. The strain 61B9, which does not express nitrogenase activity in culture, failed to produce colour in the presence of 6-cyanopurine.

Transfer of P plasmids

Plasmid R68.45 has been used to mobilize and map the chromosomes of some fast-growing strains of rhizobia (Beringer & Hopwood, 1976; Kondorosi *et al.*, 1977; Beringer *et al.*, 1978) and to construct R prime factors (Johnston *et al.*, 1978 *b*). Plasmid pRD1 is a co-integrate of RP4 and the *his–nif* region of *K. pneumoniae* (Dixon *et al.*, 1976) which has been used in complementation experiments to define the *nif* cistrons and also in several intergeneric transfers of *nif* genes to study nitrogen fixation in new hosts.

Seven strains of cowpea rhizobia were tested for ability to receive pRD1 and R68.45 from *E. coli* (Table 2). In transfer experiments, counterselection of donor strains exploited the naturally high levels of resistance in these cowpea strains to chloramphenicol and nalidixic acid to both of which *E. coli* is sensitive. Plasmid-bearing kanamycin-resistant transconjugants were selected from strains CB1024, QA922, 32H1, CB756 and a spectinomycin-resistant (Spc^r) derivative of CB756 at frequencies of 2×10^{-4}–2×10^{-6} per recipient cell. No transconjugants appeared when strains 61B9 or CB1015 were tested as recipients.

Expression of plasmid drug resistance genes

Both plasmids R68.45 and pRD1 carry genes for resistance to the antibiotics kanamycin, tetracycline and carbenicillin. Preliminary experiments suggested that CB756 transconjugants expressed kanamycin resistance most effectively while resistance to the other two drugs was less marked. Furthermore, the natural levels of resistance to these drugs are much higher in strain CB756 than in, for example, enteric bacteria. In order to quantify expression of plasmid drug resistance in CB756 transconjugants, three types of experiments were performed. The minimal inhibitory concentration (m.i.c.) of each drug was measured for strains CB756, CB756(pRD1) and CB756(R68.45) in/on medium containing various concentrations of antibiotics by: (i) efficiency and size of colony formation from diluted single cells on modLNB5 agar plates; (ii) extent of growth in liquid cultures; (iii) replica-plating colonies to solid medium (Table 3). The last method was best for demonstrating transconjugant resistance to both tetracycline and carbenicillin while kanamycin resistance was easily detected by all three methods and, in fact, served as the only marker by which transconjugant colonies could be effectively selected after transfer. Both pRD1 and R68.45 transconjugants showed identical levels of drug resistance in these experiments.

For comparison, results of a liquid m.i.c. experiment using an *E. coli* strain with and without pRD1 are shown (Table 3); they demonstrate the effective expression of all three plasmid-borne antibiotic resistance determinants.

Table 3. *Plasmid-determined antibiotic resistance in Rhizobium sp. CB756(pRD1) and CB756(R68.45)*

	Minimal inhibitory concentration of antibiotic* ($\mu g\ ml^{-1}$)								
	For colony formation			In liquid medium			By replica plating		
Strain	Km	Tc	Cb	Km	Tc	Cb	Km	Tc	Cb
CB756	75	100	60	25	200	40	100	200	75
CB756(pRD1) ⎫ CB756(R68.45) ⎭	>500	150	60	>500	250	60	>500	400	100
E. coli J62				4	2	40			
E. coli J62(pRD1)				150	30	>1000			

* Km, kanamycin; Tc, tetracycline; Cb, carbenicillin.

Plasmid stability

CB756 transconjugants were grown for 50–100 generations in antibiotic-free medium; during this time no loss of plasmid drug resistance markers was detected by replica-plating colonies to medium containing antibiotic. These results were confirmed by transfer experiments from CB756 transconjugants to a *K. pneumoniae* strain where equal and high transfer frequencies (5×10^{-2}) were measured for the three drug resistance markers during all stages of growth of CB756 transconjugants. Thus, the plasmid-borne drug resistance and transfer (*tra*) genes were stably maintained in CB756 despite the fact that both pRD1 and R68.45 increased the doubling time of CB756 from 3 h to 5 h in modLNB5 medium.

In contrast to the remarkable stability of P plasmid drug resistance genes in strain CB756, the *his–nif* region of pRD1 was rapidly lost in CB756(pRD1) transconjugants. Since neither Nif⁻ nor His⁻ mutants of CB756 were available for these studies, maintenance of *his* and *nif* pRD1 genes could only be determined in transfer experiments to suitable mutants of *K. pneumoniae*. As described above, the transfer frequency of drug resistance genes was greater than 1×10^{-2} and so could also be detected by replica-plating CB756 transconjugants to a lawn of *K. pneumoniae* CK263 (*hisD2 nifA2263*) recipients spread on a mixture of 80% modLNB5 and 20% NA medium. After incubation for 3 d, the growth was replica-plated to suitable counterselective medium. In these experiments, all mated CB756(pRD1) colonies gave rise to a patch of CK263 kanamycin-, tetracycline- and carbenicillin-resistant transconjugants but only rarely was transfer of *his* or *nif* detected. When freshly selected CB756(pRD1) colonies were tested, only 3% transferred *his* and *nif* genes to strain CK263. After subsequent non-selective growth of CB756(pRD1) for 10–20 generations, only one colony was detected among the 1500 tested that would yield CK263 His⁺ and Nif⁺ transconjugants, while drug resistance was transferred from every CB756(pRD1) colony.

Nif expression in CB756 transconjugants: nitrogenase activity in culture and effectiveness of nodules

CB756 transconjugants were tested for ability to reduce acetylene by the soft agar overlay method. The onset of nitrogenase activity was significantly delayed in plasmid-bearing organisms and activities were lower than in plasmid-free CB756 especially in CB756(R68.45). Both transconjugant types formed pink bands after incubation with 6-cyanopurine.

Symbiotic properties of transconjugants were assessed after inoculation of Siratro seedlings. In two different experiments, CB756 transconjugants formed nodules which were significantly less effective than the plasmid-free parent as measured by foliage dry weight. Transconjugants of two other cowpea strains were similarly tested and gave mixed results

Table 4. *Dry weight of shoots of Siratro inoculated with three strains of Rhizobium sp. with or without plasmids (nodulation experiment 2)*

Rhizobium sp.	Dry weight (mg)		
	No plasmid	pRD1	R68.45
CB756	50·8[ab]	38·9[c]	37·4[c]
QA922	40·7[c]	41·3[c]	26·7[d]
CB1024	55·0[a]	46·7[b]	51·0[ab]
Uninoculated	20·9[e]		

Each entry represents the mean of 10 single colonies × 8 plant replications, i.e. 80 plants. Results with the same letter do not differ significantly: least significant difference = 4·98 ($P < 0.05$).

(Table 4): the effectiveness of CB1024 was slightly, but significantly, reduced by pRD1; QA922 showed substantially reduced effectiveness when harbouring R68.45, but not with pRD1. Bacteria were re-isolated from surface-sterilized, crushed nodules and tested for retention of P plasmid by scoring growth on medium containing antibiotic. Natural high levels of drug resistance in plasmid-free QA922 made the results from nodule re-isolates ambiguous, but for CB1024 and CB756, all organisms isolated from nodules infected with plasmid-containing strains retained high levels of resistance to tetracycline, distinguishing them from plasmid-free parent nodule re-isolates.

DISCUSSION

The aim of the work presented here was to survey various techniques for usefulness in studying the function and expression of *nif* genes in *Rhizobium* sp. (cowpea strains). To that end, the most significant results are that 6-cyanopurine can be used as an indicator of *nif* expression in strain CB756, and that P plasmids are easily manipulated in several cowpea strains but may depress growth rates and *nif* expression both in culture and in root nodules.

6-Cyanopurine (CPU) was shown to be a colour indicator for *nif* expression in *K. pneumoniae* (MacNeil & Brill, 1978); Nif$^+$ colonies become dark pink after several days' incubation on nitrogen-free solid medium containing the compound. In general, mutations in the *nifAL* region which are *nifA* in complementation tests cause a CPU$^-$ phenotype but those in other *nif* genes, including the structural genes for nitrogenase, do not. The ability of strain CB756 to become dark pink in the presence of 6-cyanopurine suggests that it contains a *nif* gene product analogous to that of *nifA* which in *K. pneumoniae* is necessary for expression of the other *nif* operons. Therefore, mutants altered in degree of colour formation in the presence of 6-cyanopurine may help elucidate the mechanism of *nif* regulation in *Rhizobium* spp.

The frequency of transfer of the P plasmids pRD1 and R68.45 from *E. coli* to several slow-growing cowpea strains was comparable to that found for transfer to fast-growing rhizobia, while for two strains no transfer was detected. By comparison, Kuykendall (1979) found that P plasmid transfer from *E. coli* to *R. japonicum* occurred, if at all, at very low frequency, after mating strains on a *Rhizobium* medium. Our use of 'mixed media' (Beringer, 1974) to allow good growth of both donor and recipient during the mating period may have been a significant factor in obtaining higher transfer frequencies. In addition, counter-selection of donor *E. coli* strains was simplified by the use of drugs to which the rhizobia had a natural high level of resistance (in this case, nalidixic acid and chloramphenicol). Multiple antibiotic resistance is often found in *Rhizobium* spp. (Pattison & Skinner, 1974; Cole & Elkan, 1979; Josey *et al.*, 1979) so intrinsic drug resistance should be generally useful for counterselection of donor strains after transfer of plasmids to rhizobia.

Expression of drug resistance genes of pRD1 and R68.45 in CB756 and other cowpea strains was the same: very good for kanamycin, medium for tetracycline and poor for

carbenicillin (ampicillin). Similar patterns of expression have also been noted for P plasmids in *R. leguminosarum* (Beringer, 1974; Nagahari *et al.*, 1979; Ram & Kumar, 1979) and in *R. japonicum* (Kuykendall, 1979) but ampicillin resistance was significant in *R. lupini* harbouring RP4 (Pühler & Burkardt, 1978).

R68.45 and pRD1 drug resistance and *tra* genes were stable in CB756 transconjugants; no plasmid loss was detected after many subcultures in non-selective medium despite the fact that growth rates of CB756 were almost halved in transconjugant clones thereby imposing theoretically strong selection for plasmid-free segregants. Similar stability of P plasmid drug resistance genes has been observed in *R. leguminosarum* (Beringer, 1974) and *R. meliloti* (Spitzbarth *et al.*, 1979), while in *R. lupini* (RP4) both drug resistance and *tra* genes are frequently lost after non-selective growth (Pühler & Burkardt, 1978).

Plasmids were maintained in CB756 even after passage through root nodules since bacteria isolated from surface-sterilized, crushed nodules were tetracycline-resistant (no effort was made to ensure bacteroid viability and the re-isolated organisms could have come from any stage of infection). Tests of effects of introduced plasmids on nodulation properties of rhizobia have been reported for only one species: Spitzbarth *et al.* (1979) found no effect of RP4 on nodulation or effectiveness in *R. meliloti* infecting *Medicago sativa* L. Surprisingly, however, RP4 restored effectiveness to an ineffective mutant as shown by nodule acetylene reduction. We found differences among the strains tested: with CB756 the presence of either pRD1 or R68.45 reduced symbiotic nitrogen fixation (and acetylene reduction in culture); with QA922 nodule nitrogen fixation was reduced by one plasmid but not by the other, while with CB1024 the converse situation occurred. On the other hand, the *his–nif* region of pRD1 originally derived from *K. pneumoniae* was lost at high frequency from the plasmid as determined by the failure of CB756(pRD1) to transfer *his–nif* along with drug resistance to appropriate *K. pneumoniae* recipients. Thus *K. pneumoniae nif* expression in CB756 could not be assessed; to do so it would be necessary to have a His⁻ mutant for selective growth of *nif*-bearing transconjugants. pRD1 is notorious for losing the *his–nif* region during non-selective growth in diverse organisms such as *Azotobacter vinelandii* (Cannon & Postgate, 1976), *R. meliloti* (Dixon *et al.*, 1976) and *Salmonella typhimurium* (Postgate & Krishnapillai, 1977).

The reduction by P plasmids of growth rates of CB756 is surprising and, as far as we know, an effect so great has not been reported for other bacteria although small changes have been observed (for example, Dale & Smith, 1979).

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