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Short Communication

Three Additional Loci of Rifampicin Dependence in *Escherichia coli*

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Summary. Rifampicin dependent mutants had been isolated as conditional lethal mutants with a lesion affecting transcription. Substituting Tn10 coded tetracycline resistance as a stable marker instead of the unstable rifampicin dependence facilitated mapping of dependence mutations. Three new *rid* loci were defined in this way. *ridB* and *ridD* were on opposite sides of *ilv* at about 84 min, and *ridC* was at about 35 min on the *Escherichia coli* chromosomal linkage map. Two more mutants with the previously described *ridA* lesion were also found. Growth response to removal of rifampicin was different for *ridA, B,* or *C* strains. Double mutants were constructed, and in all cases the more limiting phenotypic response prevailed.

I have previously reported the isolation of mutants of *Escherichia coli* dependent on the presence of the antibiotic rifampicin in medium for growth (Dabbs 1979). Rifampicin acts by binding to RNA polymerase and inhibiting transcription (Bähr et al. 1976); it inhibits initiation of transcription (Johnston and McClure, 1976). Rifampicin dependent (Rif-D) mutants were isolated because it was anticipated these might have acquired a conditional lethal mutation in a cellular component involved in the transcription process. Genetic analysis (Dabbs 1979; Dabbs and Looman 1981, this work) has in every case investigated shown the phenotype to be the product of a rifampicin resistance (Rif-R) *rpoB* mutation together with a dependentizing mutation located elsewhere on the chromosome.

Mapping of dependentizing mutations has been hindered by instability of the dependent phenotype. A reversion frequency usually in the range of 10^{-3} to 10^{-4} resulted in antibiotic independent secondary mutants forming a significant portion of any culture. Mapping required Hfr crosses in which recombinants were scored for loss of dependent phenotype but, due to reversion, there was a fraction of recombinants that were no longer dependent irrespective of the Hfr strain used.

The opportunity to replace an unstable dependence mutation by a stable resistance mutation is offered by λ phages carrying transposable resistance elements (e.g. Kleckner et al. 1978) which could be used to insert a transposon at a variety of locations in the chromosome. In this paper, I describe the mapping of three loci of rifampicin dependence using Tn10 insertions. These are loci additional to that previously reported (Dabbs and Looman 1981). The properties of genetically defined strains were investigated, and the transposon mediated resistance marker was used to observe the outcome of combining non-allelic Rif-D mutations.

Methods were as previously described (Dabbs 1979; Dabbs and Looman 1981). Strains used in this work are listed in Table 1. To obtain a pool of tetracycline resistant (Tc-R) strains, λNK370 (*b*221 *c*1857 *c*1171::Tn10 *O*uga261) was used to infect strain MA1030 which was then plated on medium containing 20 μg per ml tetracycline (Tc) and incubated at 37° C. About 1100 Tc-R clones were pooled and a P1 lysate prepared of the mixture.

Rif-D mutants were chosen from amongst strains previously isolated for their possession of a diversity of phenotypes. To select Tn10 insertions near the Rif-D loci, I used the P1 lysate of the pool of Tc-R strains as donor and the dependent strains as recipients. After incubation with the P1, the cells were spread on plates containing Tc but lacking rifampicin (Rif). For each mutant the transduction was repeated several times, with qualitatively the same result for a particular mutant. No Tc-R transductants grew up in the case of three mutants tested. However, transductants were obtained from strains PB10, PB12, PB17, PB61, and PB70.

Contransduction of Tc-R with Rif-D was confirmed for each dependent mutant, and the tetracycline marker was selected into strain TA10. This was done to circumvent the inability of some mutants to grow on minimal medium supplemented with the requirements of the parental strain, presumably due to lesions arising during the mutagenesis used in the selection procedure. Hfr crosses were made with the Tc-R transductants of strain TA10.

PB61. The Tn10 insertion isolated from this mutant was placed near *ilv* at about 84 min, on the basis of gradient of transmission experiments with Hfr strains KL209 and KL14. P1 transductions gave the result that Tc-R was about 80% contransducible with *ilv* of strain AB2147. This Tc-R marker was also selected into mutant PB61, with the

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Table 1. Strains used in this work

Strain	Phenotype and genotype	Source reference
AB2147	F⁻ *ilv192 (A or Y) argH1 metB1 his-1 tna-1 thi-1 xyl-4 malA1 lacY1 gal-6 rpsL tsx-7*	S. Pedersen
AT2444	Hfr *metE68 thi-1 relA1 spoT1* (PO 1)	B. Bachmann
CP78	F⁻ *thr-1 leuB6 his-65 argH46 thi-1 gal-3 malA1 xyl-7 mtl-2 tonA2 supE44*	K. Isono
GMS343	F⁻ *argG3 aroD6 lacY1 galK2 mtl-1 xyl-5 man-4 rpsL700 tsx-29 supE44*	K. Isono
KL14	Hfr *thi-1 relA1* (PO 68)	K. Isono
KL96	Hfr *thi-1 relA1 spoT1* (PO 44)	B. Bachmann
KL99	Hfr *thi-1 relA1 spoT1 lac-42* (PO 42)	B. Bachmann
KL208	Hfr *relA1* (PO 43)	B. Bachmann
KL209	Hfr *malB16* (PO 18)	K. Isono
MA1030	Hfr *argR46 purF1 thi-1* (PO 12)	B. Bachmann
PK191	Hfr *thi-1 relA1 supE44* (PO 66)	B. Bachmann
TA10	F⁻ as strain CP78, *aroE24 rpsE gyrA*	(Dabbs and Looman 1981)
PB10, 12, 17	as strain CP78, *rpoB310 ridA2, rpoB312 ridD1, rpoB317 ridA3* respectively	(Dabbs 1979)
PB61, 67, 70	as strain TA10, *rpoB361 ridB1, rpoB67 ridA1, rpoB370 ridC1,* respectively	(Dabbs and Looman 1981)
PB102, 122, 612,702	as strain TA10, with Tn10 insertion (this work) from strains PB10, 12, 61, 70 respectively	

λNK370 (*b221 c1857 c1171*::Tn10 *O*uga261) was provided by B. Mygind

outcome that 30 of 112 (27%) of the clones were no longer dependent. The locus of this Rif-D mutation near *ilv* was termed *ridB1* (for *ridA*, see Dabbs and Looman 1981).

A lysate of a Rif-D Tc-R transductant obtained in the previous selection was donor of the Tc marker into strain TA10. No Rif-D transductants were obtained so I concluded that the *ridB1* mutation was necessary but not sufficient for the phenotype. Since I believe that *rid* (*ri*fampicin *d*ependent) lesions alter the response of RNA polymerase to Rif, a likely candidate for the other mutation necessary for the phenotype to be expressed was the Rif-R *rpoB* mutation of strain PB61. When an *rpoB361* transductant of strain TA10 was recipient to Tc-R from the same lysate as used previously then about 15% of the clones showed the Rif-D phenotype of mutant PB61. Therefore the *ridB1* and *rpoB361* mutations both had to be present in order for the dependent phenotype to be expressed. In contrast, the *ridA* lesion (Dabbs and Looman 1981) alone was sufficient to confer the Rif-D phenotype on a strain.

To place *ridB* and the Tn10 insertion in the context of established markers in this region of the linkage map (Bachmann and Low 1980) I performed other transductions. A Tc-R transductant of mutant PB61 was donor of either *ilv*⁺ or Tc-R into an *rpoB361* derivative of strain AB2147. The segregation of markers (Table 2) indicated that *ridB* and Tc-R were on opposite sides of *ilv*. The nearby *tna* marker was also used. More *tna*⁺ transductants were Tc-R than were *ilv*⁺ (Table 2) but none were *ridB1*. Therefore, the likely order was *tna*-Tn10-*ilv-ridB*. *ilv* and *ridB* were about 45% contransducible. Experiments with *metE* strain AT2444 agreed with other results in giving the order Tn10-*ridB-metE*. *ridB* and *metE* were about 28% cotransducible.

PB10 and PB17. The Tn10 insertion obtained from mutant PB10 was placed near *aroE* on the basis of gradient of transmission experiments using Hfr strains KL14 and KL209. This was confirmed when a streptomycin resistant (Sm-R) *rpsL* mutation was transduced into strain PB102. About 70% (35 of 49) Sm-R transductants were no longer Tc-R. A Tc-R transductant that was *rpsL rpsE aroE* was

Table 2. P1 mediated crosses between *tna*, Tc-R, *ilv*, and *ridB*

Cross	Selected marker (no. scored)	Transductant characteristics[a]				Number of transductants (% of total)
		tna	Tc	*ilv*	*ridB*	
1. Tc^r *ilv*$^+$ *ridB1* (donor) Tc^s *ilv192 ridB*$^+$ (recipient)	Tc^r (104)		r	+	+	51 (49)
			r	+	−	28 (27)
			r	−	+	33 (32)
			r	−	−	2 (2)
2. Tc^r *ilv*$^+$ *ridB1* (donor) Tc^s *ilv192 ridB*$^+$ (recipient)	*ilv*$^+$ (188)		r	+	+	79 (42)
			r	+	−	68 (36)
			s	+	+	20 (11)
			s	+	−	21 (11)
3. *tna*$^+$ Tc^r *ilv*$^+$ (donor) *tna* Tc^s *ilv192* (recipient)	*tna*$^+$ (62)	+	r	+	[b]	23 (37)
		+	r	−		11 (18)
		+	s	+		0 (0)
		+	s	−		28 (45)

[a] "+" wildtype; "−" mutant allele. "r" resistant; "s" sensitive allele
[b] *ridB* was also scored; all transductants were *ridB*$^+$

recipient of *aroE*$^+$ from strain CP78; the marker segregation pattern indicated that the transposon had been inserted between *rpsL* and *rpsE*.

When Tc-R was selected from strain PB102 into Rif-D mutant PB10 only 9 of 460 colonies (2%) had lost the dependentizing mutation. This figure, and the pattern of segregation of markers among transductants, was compatible with the Rif-D lesion residing at the previously characterised *ridA* locus (Dabbs and Looman 1981). In other crosses, the lesion was 5–8% cotransducible with *aroE*. No *rid*$^+$ recombinants were obtained in crosses between mutant PB10 and *ridA1* mutant PB67, which was support for the allelic nature of the mutations in these strains. Screening of other Rif-D mutants turned up one additional strain which probably had a mutation at the *ridA* locus; PB17.

PB70. The Tn10 insertion derived from this mutant was covered by Hfr strain KL99 but not by Hfr strains PK191 or KL208. Therefore the insertion was between 30 and 42 min on the *E. coli* linkage map. Introduction of Tc-R from strain PB702 into mutant PB70 led to loss of dependent phenotype in about 40% of transductants. When a Tc-R Rif-D transductant from this cross was donor of tetracycline resistance, about 15% of transductants acquired a Rif-D phenotype.

Strain GMS343 (Table 1) possessed an *aroD* mutation located just clockwise to the marker-poor region of the map around the chromosomal replication terminus. Strain GMS343 was rendered *his* by auxotroph enrichment and the Tc-R marker of strain PB702 used to introduce the dependent mutation. Hfr crosses were made using strains KL96 and KL99, selecting *his*$^+$ or *aroD*$^+$ and scoring the other auxotrophy. The tetracycline and rifampicin phenotypes were also scored. These experiments positioned the lesion, termed *ridC*, near *aroD*. However, in P1 transduction experiments I was unable to show linkage of *aroD* and *ridC* or Tc-R.

PB12. A Tn10 insertion obtained from mutant PB12 was found to have entered the chromosome at a position similar to that of mutant PB61, i.e. near *ilv*. About 9% of *ilv*$^+$ transductants of an *rpoB312* derivative of strain AB2147 showed a Rif-D phenotype when mutant PB12 was donor of *ilv*$^+$. In a series of crosses similar to those done with mutant PB61 I found that the lesion was on the other side of *ilv* from *ridB*. The locus was termed *ridD*.

Phenotype. The phenotype of cells harboring *ridA*, *B*, or *C* mutations was compared. The response of growing cultures of mutant strains to removal of antibiotic was determined. Experiments were also done to observe the effect of combining *rid* lesions. The phenotypes of mutants PB10, PB17, PB61, and PB70 are compared in Table 3; since *ridA1* mutant PB67 had previously been shown to also grow on medium containing the ribosomally targeted antibiotic kasugamycin (Dabbs and Looman 1981), the response to this antibiotic was also observed. The phenotypes arising from the three classes of mutation were quite distinct.

ridC1 and *ridA2, 3* mutations resembled the *ridA1* mutation in that the *rid* mutation alone was sufficient to give the Rif-D phenotype. *ridB1* alone gave rise to no detectable phenotype; a Rif-D phenotype was only expressed upon introduction of the *rpoB361* mutation. Such a strain was

Table 3. Antibiotic phenotype of *ridA, B,* and *C* mutants

Strain	Rif-D mutation	Rich medium					Minimal medium[a]	
		Rif			Ksg		Rif	
		30[b]	37	42	30, 37	42	30	37
PB10	*ridA2*	D	D	D	D	D	D	D
PB17	*ridA3*	D	D	–	D	–	D	D
PB61	*ridB1*	D	D	R	–	R	R	R
PB70	*ridC1*	R	R	D	I	–	R	R

Antibiotic concentrations: 10 μg per ml Rif. 60 μg per ml Ksg (wildtype *E. coli* strains are not inhibited by this concentration). D, R, I = antibiotic dependent, resistant, inhibited. – = no growth in either the presence or absence of antibiotic

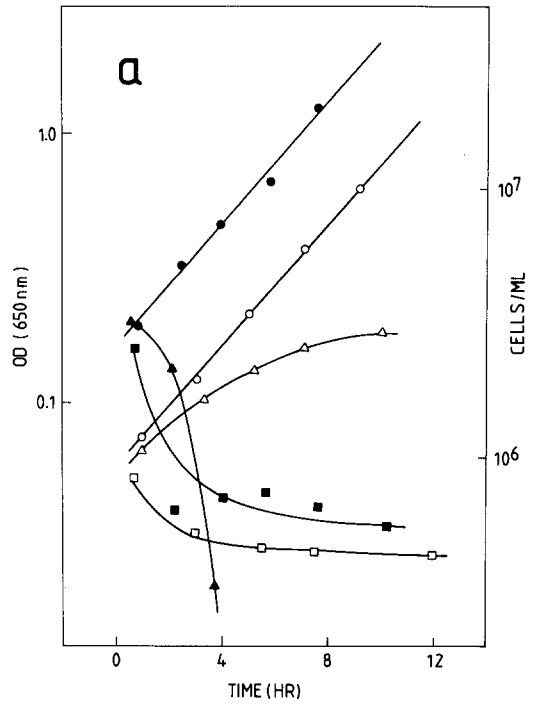
[a] Strain CP78 and its derivatives are unable to grow well at 42° C on minimal medium

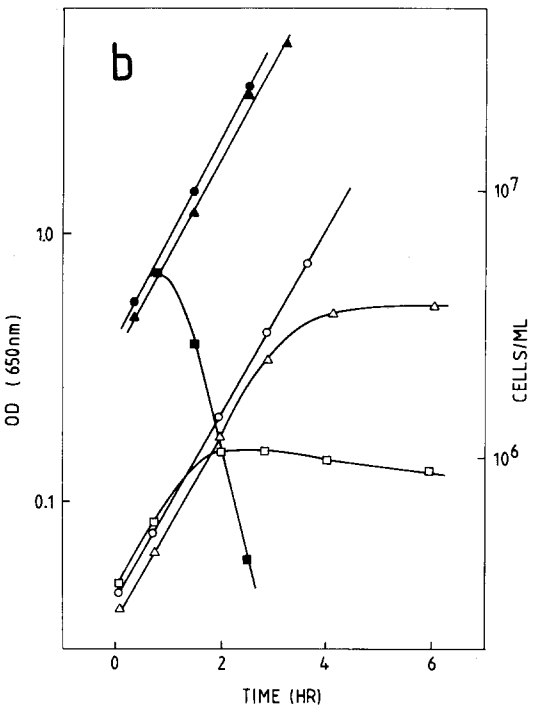
[b] °C

Rif-D on rich medium below 40° C but not Rif-D on minimal medium at any temperature. Presence of *ridA* mutations led to a Rif-D phenotype on both rich and minimal medium. In an otherwise isogenic context, the phenotype arising from the *ridA2* lesion was indistinguishable from that due to *ridA1*, but in terms of doubling time *ridA3* was distinguishable from both the other alleles.

Antibiotic Growth Response. I measured the response to antibiotic removal with *rpoB361* derivatives of strain CP78 harboring either the *ridA2*, *ridB1*, or *ridC1* mutation. The results are shown in Fig. 1. There is a contrast in the behavior of the *ridA* and *ridB* strains. In the former, after resuspension of cells in medium lacking Rif there was continued increase in cell number and mass for at least one generation time, followed by cell death. Antibiotic removal from the *ridB* strain was rapidly followed by death of 60–70% of cells but thereafter the viable count was stable. After the same treatment, the *ridC* strain showed increased viable count and optical density but at a diminishing rate. Because kasugamycin can sustain growth of *ridA* strains when Rif is not present (Dabbs and Looman 1981), I determined what happened to *ridB* and *ridC* strains exposed to the same conditions. In both cases, substitution of kasugamycin resulted in cell death (Fig. 1).

rid Mutations Combined. Additional information about the nature of the cellular components altered by mutational lesions can be provided by combining them. The insertion of the Tc-R marker near the mutant loci facilitated the transfer of mutations into other Rif-D strains. *ridA2 ridB1*, *ridA2 ridC1*, and *ridB1 ridC1* double mutants were constructed (in all cases a CP78*rpoB361* background was used). The *ridB ridC* double mutant was dependent on Rif in rich medium at all temperatures (cf. Table 3 for the individual mutations) not Rif-D in minimal medium and unable to grow on rich medium supplemented with kasugamycin. The *ridA ridC* had a similar phenotype except that it was also Rif-D on minimal medium. With every combination of medium, temperature, and antibiotic, for these double mutants the more limiting phenotypic alternative prevailed. There was no evidence of blending or averaging of phenotype





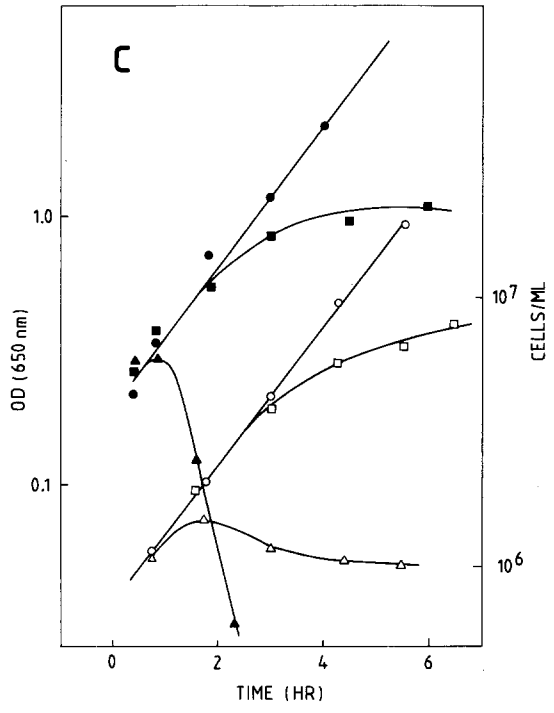


Fig. 1a–c. Growth curves of *rpoB361*CP78 strains harboring: (**a**) *ridB1* (**b**) *ridA2* (**c**) *ridC1* mutations. In (**a**) and (**b**) the temperature was 37° C, in (**c**) 42° C. OD (650 nm) in presence of 10 μg per ml Rif (○), in absence of antibiotic (□), in presence of 60 μg per ml Ksg (△). Viable counts in presence of 10 μg per ml Rif (●), in absence of antibiotic (■), in presence of 60 μg per ml Ksg (▲). Cells growing in Rif containing medium were washed and resuspended in medium for the experiment at 0 h. 10 μg per ml Rif was present in medium used for pregrowth of cells and in plates used to measure viable counts. Viable counts were also measured on plates not containing any antibiotic; the counts were in all cases about 10^{-5} to 10^{-4} of those from Rif containing plates and represented antibiotic independent secondary mutants

(as manifested for example by doubling time or reversion rate) nor any evidence of novel phenotype.

The properties of the *ridA2 ridB1* was Rif-D on minimal medium and unable to grow on medium supplemented with kasugamycin, so again the more limiting condition prevailed. However, a novel phenotype was presented by the observation that this double mutant could only grow at 42° C (contrast Table 3). This indicated the possibility that there was an interaction between the products of the *ridA* and *ridB* genes.

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