A RAPID TEST FOR THE REL A MUTATION IN E. COLI

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Summary : rel$^+$ and rel$^-$ K12 strains from E. coli can be separated on liquid or solid media, according to their ability to grow on excess one-carbon metabolites. Minimum medium supplemented with serine, methionine, and glycine permits growth of rel$^+$ but not of rel$^-$ strains.

In E. coli, protein synthesis is strongly coupled to stable RNA synthesis owing to a mechanism which has not yet been completely elucidated. Several genes have been shown to be involved in this coupling [1]. The relA gene is expressed as follows : when protein synthesis is restricted after a large decrease in some amino acid availability (e.g. in an auxotrophic strain, or after an overdose of valine which leads to isoleucine starvation), the synthesis of stable RNA is almost immediately shut down, with the concomitant appearance of the nucleoside tetraphosphate ppGpp (stringent response). In a relaxed mutant, RNA synthesis proceeds for a long time while ppGpp is not formed.

In order to test for a relaxed or stringent response it is therefore necessary to assay for nucleoside incorporation into RNA, in parallel with amino acid incorporation into protein. This is a rather tedious procedure, especially when one has to test for the relA gene in a number of different mutants. In addition, this general method does neither provide means for studying the genetics of relA (e.g. isolation of revertants) nor enable one to isolate transducing phages carrying this interesting gene.

In the course of our study on secondary punctuation in prokaryotes (centered on the various signals for the initiation of translation [2], we have observed that relaxed and stringent strains exhibit different behaviours. The main observation is that growth of rel$^-$ strains is completely inhibited at 37°C by the one-carbon pool metabolites, whereas rel$^+$ bacteria remain almost unaffected. This allows a very easy characterization of the relA mutations on plates or liquid media, and opens a number of further studies.

Abbreviations : rel$^-$: relaxed strain
rel$^+$: stringent (wild type) strain
SMG : serine + methionine + glycine

We shall here describe the test and summarize the effect of several media on well-known relaxed strains.

MATERIALS AND METHODS

Escherichia coli K12 strains come either from the Institut Pasteur Collection, or from the E. coli Genetic Stock Center, thanks to the courtesy of Dr. B. Bachman. Three different alleles of relA were used :

Rel-1 Hfr : H (rel-1, thi-1) KL 14 (rel-1, thi-1), KL 16 (rel-1, thi-1), KL 16-99 (rel-1, recA, drm, thi-1), AT 2427 (cysC, thi-1, rel-1), AT 2457 (glyA6, rel-1, thi-1), and AT 2457 G is the glyA$^+$ strain obtained by P_1 transduction from the preceding one. KL 16 having lost the F factor (F^-) has also been assayed.

rel CP79 [3] CP79 (F^-, thr, leu, arg, his, rel$^-$), and the parental CP78 (F^-, thr, leu, arg, his, rel$^+$).

rel PA_1 [2, 4] PA_1 (arg, str, lac, rel$^-$), and the parental PA_2 (arg, str, lac, rel$^+$).

Amino acid and nucleotide incorporation has been measured on exponentially growing cultures, as usually described [5]. The relaxed mutation has been expressed in auxotrophic strains by starving for a required amino acid. In prototrophic bacteria, excess valine (200 µg/ml) has been added. This very rapidly starves the cell for isoleucine and yields the same results as in an auxotrophic strain.

Plates or liquid media were prepared by using minimum medium M63 [6] supplemented with thiamine (5 µg/ml) and the required amino acids (100 µg/ml of the L-form). Other metabolites were added, as described in the text. We generally used glucose (0.4 %) as a carbon source. In some experiments glucose was replaced by glycerol (0.6 %).

RESULTS AND DISCUSSION

Inhibition by serine + methionine + glycine.

Figure 1 shows the original observation that the one-carbone pool metabolites (serine, glycine, methionine : 100 µg/ml each ; adenine : 50 µg/ml ; thymine : 20-50 µg/ml ; calcium pantothenate : 1 µg/ml) completely inhibit the growth of rel$^-$ strains on minimal plates leaving rel$^+$ strains almost completely unaffected.

In order to use this phenomenom as a simple test for the relA gene, we studied growth inhibition of the rel$^-$ strains by the one-carbon pool derivatives separately either in liquid or on solid media. Serine alone can inhibit rel$^-$ mutants, but only at high concentration ($\geqslant$ 150 µg/ml) ; at 100 µg/ml growth is not inhibited, but the generation time is doubled. However, serine acts as a trigger which can be used for discriminating rel$^+$ from rel$^-$ if

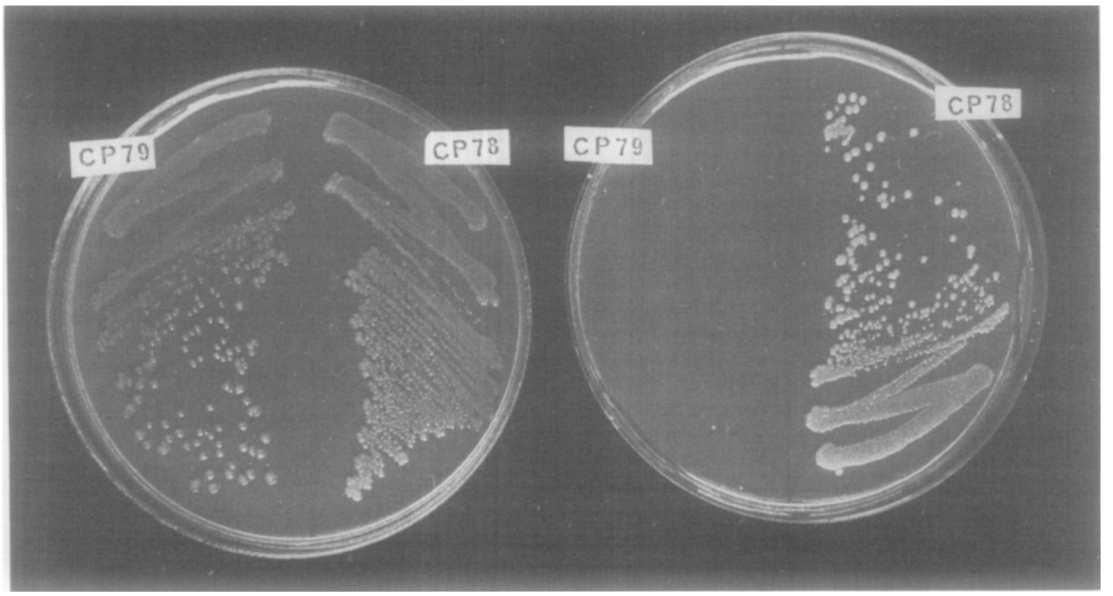

Fig. 1 - Comparison between rel$^+$ and rel$^-$ strains growing on excess one-carbon metabolites (37°C).
Left : medium M63 supplemented with glucose (0.4 %), thiamine (5 µg/ml) and L-threonine, L-leucine, L-arginine, L-histidine (100 µg/ml each) : CP 78 (rel$^+$) and CP 79 (rel$^-$) grow equally well.
Right : same medium supplemented with the one-carbon pool metabolites (see the test) : growth of CP 78 is almost normal whereas that of CP 79 is completely inhibited.

another amino acid from the one-carbon pool is added. Figure 2 shows that in minimal liquid medium, when either methionine or glycine, or both are added beside serine, the growth of rel$^+$ and rel$^-$ strains is inhibited. However, after a lag of 4-5 hours at 37°C, the rel$^+$ strain resumes its normal growth (Fig. 2a), whereas the rel$^-$ strain remains inhibited for 8 hours (Fig. 2b). In this experiment, the starting cell concentration was about 10^7 cells/ml. If the cultures are one hundredfold diluted (about 10^5 cells/ml), growth inhibition of rel$^-$ strains in SMG medium is maintained for at least 48 hours. This effect of cell concentration will be considered later. Serine + methionine + glycine (SMG) act in a synergistic manner since we find that the minimum inhibitory dose is as low as 10 µg/ml of each amino acid (3.2 % growth of CP79 after 32 hours in SMG, 10 µg/ml each, as compared to growth without SMG). Conversely, rel$^+$ strains are highly resistant since addition of SMG (150 µg/ml each) do not yield any difference in the stationary amount of cells after 24 hours, as compared to that obtained in the absence of SMG.

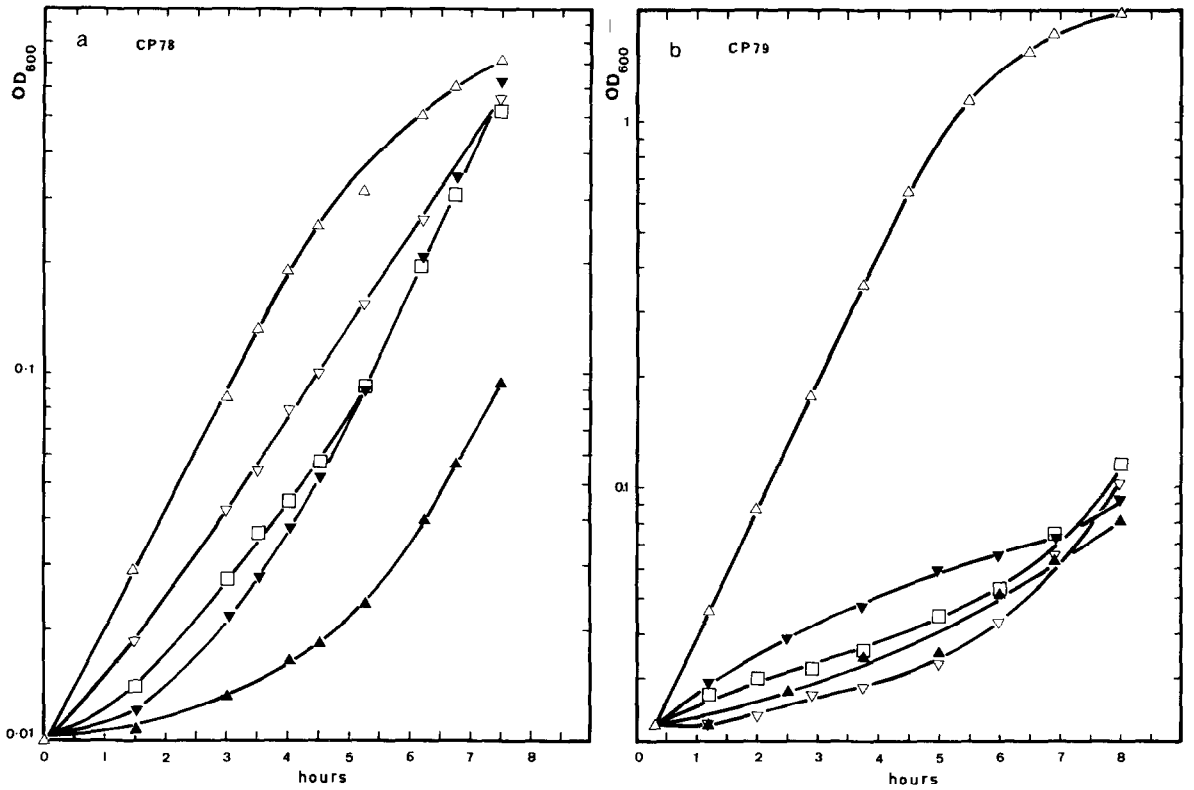

Fig. 2 - Growth curves of CP 78 and CP 79 influenced by serine, methionine and glycine.
Exponentially growing cultures of CP 78 (2a) and CP 79 (2b) in minimal medium supplemented with 100 µg/ml each of L-threonine, L-leucine, L-histidine, and L-arginine, were divided into five subcultures, each containing 100 µg/ml of the following amino acid : (△) none ; (▽) L-serine ; (□) L-serine + L-glycine ; (▼) L-serine + L-methionine ; (▲) L-serine + L-methionine + L-glycine. Cell growth is followed by monitoring the optical density at 600 nm on a Spectrophotometer.

On solid media we tested the relA gene by streaking a dilution of the bacteria on minimal plates containing SMG at 100 µg/ml for each amino acid. The same inhibition of rel⁻ mutants is obtained whether glucose or glycerol is used as carbon source when SMG is added onto the plates. Under such conditions there is no background growth after 48 hours. However, we observed that there is some influence of the cell concentration on residual growth : some residual growth of relaxed mutants can be observed if one uses a large number of cells. One should avoid the spreading of more than 10^7 cells per plate (10 cm in diameter). Similarly one should not, in liquid media, start from inocula corresponding to cell

TABLE I

Inhibition of growth by SMG (1:1:1) for two relA alleles.

		µg/ml of each amino acid					
		0	10	20	50	100	150
		relative growth after 32 hrs (inoculum : 10^4 cells)					
CP 78	(rel$^+$)	100	100	100	100	100	100
CP 79	(rel$^-$)	100	3	0	0	0	0
AT 2457 G	(rel$^-$, glyA$^+$)	100	90	0	0	0	0
AT 2457	(rel$^-$, glyA$^-$)	100	100	95	15	2	0

concentrations higher than 10^7 cells per milliliter. Besides, as we shall see later, the mutations which allow the growth of relaxed strains on SMG are quite numerous. This can interfere with the test and we prefer to use the streaking technique rather than replica plating. The latter can nevertheless be used provided that the replica is very light.

Under such conditions all the rel$^-$ strains that we have tested (listed in MATERIALS AND METHODS) were completely inhibited (except for AT 2457 : see later). Conversely, all rel$^+$ strains grow normally without being affected.

Other inhibitory metabolites : involvement of the folate metabolism.

Although our purpose in the present article is only to describe a new technique for differentiating rel$^+$ from rel$^-$ strains, we shall now present some evidence for the primary mechanism which leads to inhibition of the growth of the rel$^-$ strains. A more thorough description of the chain of inhibitory events will be given elsewhere : a preliminary account can be found in Ref. 7.

Since we observed that serine occupied a central position in the inhibition of rel$^-$ mutants and since the one-carbon pool metabolites appear to be involved, we assayed the influence of SMG on a rel$^-$ strain devoid of serine hydroxymethyl transferase [8] (glyA : auxotrophic for glycine or threonine). This enzyme (missing in the glyA mutant) catalyses the reaction

TABLE II

Growth of *rel*$^+$ and *rel*$^-$ strains in the presence of serine (100 μg/ml) together with other metabolites.

	growth of	
	CP 78 (*rel*$^+$)	CP 79 (*rel*$^-$)
methionine (100 μg/ml)	+	+
serine + methione	+	-
Vitamin B12 (2 μg/ml)	+	+
serine + vitamin B12	+	-
glycine (100 μg/ml)	+	+
serine + glycine	+	-
adenine (100 μg/ml)	+	+
serine + adenine	+	- (slow residual growth)
pantothenate (50 μg/ml)	+	+
serine + pantothenate	+	+
formate (200 μg/ml)	+	+
serine + formate	+	+
2-ketoglutarate (100 μg/ml)	+	+
serine + 2-ketoglutarate	+	-
isocitrate (100 μg/ml)	+	+
serine + isocitrate	+	-
L-malate (100 μg/ml)	+	+
serine + L-malate	+	-
cis-aconitate (100 μg/ml)	+	+
serine + cis-aconitate	+	-
oxaloacetate (100 μg/ml)	+	+
serine + oxaloacetate	+	-

The growth was observed after 24 hrs on M63 plates supplemented with glucose (0.4 %), L-threonine, L-leucine, L-histidine, and L-arginine (100 μg/ml each, and thiamine (5 μg/ml). Bacteria were streaked with a platinum loop.

Serine + Tetrahydrofolate ⟶ Glycine + $N^{5,10}$-Methylenetetrahydrofolate.

This reaction is the major entry in the one-carbon pool metabolism. In the mutant *glyA* the major entry of one-carbon residues uses glycine instead of serine as a one-carbon donor and the turnover of the one-carbon pool is slower than in the wild type (the generation time at 37°C is at least 50 % longer than that of the wild type).

Table I shows the results obtained : the glyA mutation partially reverses the influence of the rel⁻ mutation. This strongly suggests that the primary effect of SMG is to increase the level of methylenetetrahydrofolate and that this metabolite leads to an inhibition of growth of relaxed strains.

To further support this hypothesis we used a sub-inhibitory concentration of serine (100 µg/ml) and added metabolites which would tend to increase the methylenetetrahydrofolate pool : either one-carbon pool metabolites or Krebs cycle derivatives (which would increase the turnover of dihydrofolate and its conversion to tetrahydrofolate). The results, summarized in Table II, show that, as expected, most of the metabolites are inhibitors in the presence of serine, and not in its absence. The fact that formate does not inhibit suggests that its entry in the one-carbon pool is not very active.

Temperature effect.

rel⁻ strains are more or less thermosensitive and grow rather poorly on minimal plates at 42°C. We therefore were led to study the influence of temperature on the SMG effect. As described above, SMG clearly inhibits the growth of rel⁻ mutants at 37°C. It is worth noting that at 30°C, there is an appreciable growth of the rel⁻ strains, colonies having about half the size of rel⁺ colonies ; furthermore, at 25°C there is no longer any difference between the wild type and the relaxed mutants. Thus, our test is not well-suited for thermosensitive strains harbouring the rel⁻ mutation ; it can, however, still be used at 30°C if the amount of SMG is increased to 150 µg/ml each : there is still a residual growth of rel⁻ strains but these mutants can now clearly be distinguished from the rel⁺ strains.

The effect of temperature may be used as an internal control of the test : bacteria are not killed by SMG and they will grow at 25°C after having been preincubated for 48 hours at 37°C in the same medium.

In order to estimate the number of bacteria that can be plated and give no background growth, we measured the proportion of mutants derived from a rel⁻ strain which would grow on SMG at 37°C. This proportion is very high (about one in 10^5 relaxed bacteria) and depends on the composition of the medium ; the lowest proportion is obtained with 100 µg/ml each of SMG. We have checked that the colonies which grew on SMG were actual mutants : after three purifications on a medium devoid of SMG, these mutants grow on SMG as the rel⁺ strains. Because of this high mutation rate, it is necessary to use a large dilution of bacteria (streaking provides a convenient means to do so) or a very light replica.

CONCLUSION

We have described a simple test which enables to separate *rel*⁻ mutants from the *rel*⁺ *E. coli* strains. It is based on a selective and dramatic growth inhibition of the *rel*⁻ strains when they are incubated in minimum medium supplemented with the one-carbon pool metabolites. One could take advantage of this property of the *rel*⁻ mutants to transduce the *relA* gene in various strains. We should emphasize however that our test do neither show that all sensitive strains are *rel*⁻ nor that all resistant strains are *rel*⁺.

In addition, our results indicate that a signal directly derived from the folic acid metabolism could play an important role in the control of cell growth, in minimal medium at least. This result, suspected from previous work (2, 7) is now further investigated.

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