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Correlation Between the Serine Sensitivity and the Derepressibility of the *ilv* Genes in *Escherichia coli relA*⁻ Mutants

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Summary. Upon addition of excess one carbon metabolites (including serine) bacteria stop growing because of isoleucine starvation. After such treatment stringent bacteria rapidly resume normal growth whereas relaxed mutants remain unable for some time to grow. We show here that this is due to a lack of derepressibility of *ilv* genes after the starvation period. Results are also presented which show that RNA polymerase structural mutants may be selected among the clones resistant to a mixture of serine, methionine and glycine, in *relA*⁻ strains. Finally circumstancial evidence suggests that the one carbon metabolism may be involved in a process controlling isoleucine metabolism.

Introduction

Escherichia coli K 12 mutated in the stringent coupling between translation and transcription of stable RNAs have been shown to stop growing in various types of shift down experiments such as shifts from a rich carbon supply to a poor one (Fiil and Friesen, 1968) or from a synthetic growth medium supplemented with amino acids to an unsupplemented medium. In this latter case it has been observed that the presence of an excess of particular amino acids (namely pehnylalanine, methionine, leucine, isoleucine or serine) in the receiving synthetic medium greatly enhances the growth inhibition caused by the downshift transfer (Alföldi et al., 1963; Alföldi and Kerekes, 1964; Edlin and Broda, 1968).

We have recently described conditions under which *relA* mutants are unable to grow on some synthetic media, without shift down conditions. A shift up from a minimal synthetic medium to the same medium supplemented with the one carbon amino acids serine, methionine, glycine, results in a dramatic interruption of growth of both relaxed and stringent bacteria. The stringent strains resume normal growth within one to four hours whereas the relaxed strains remain completely inhibited (Uzan and Danchin, 1976). We have shown that this inhibition is triggered by serine. Besides many other metabolites such as particular amino acids, acids from the tricarboxylic acids cycle and some vitamins and purines appear to act in a synergistic manner with serine although they do not affect individually bacterial growth. This synergy enables to follow the serine effect in the concentration range of 0.1 mM–1 mM under some conditions.

An inhibitory action of L-serine, albeit at a very high concentration, 5–10 mM, on the growth of wild type *E. coli* has been previously mentioned by several authors, and mutants which exhibit hypersensitivity to exogeneous serine have been isolated; the corresponding mutations map in different places of the *E. coli* chromosome. In all cases branched-chain amino acids could reverse the inhibition (Cosloy and McFall, 1970; Raskó and Alföldi, 1971). The involvment of the *relA* gene in this inhibition process prompted us to investigate in more details the growth inhibition induced by a mixture of the one carbon amino acids (serine, methionine and glycine). In this article we describe new features of the serine effect and report genetic evidences showing that derepression of the *ilv* operons is very much impaired in a *relA* background as compared to the *relA*⁺ isogenic background, after addition of excess one carbon metabolites. This difference appears to be the basis of the extreme sensitivity to serine of relaxed mutants.

Abbreviations: Throughout this work we have represented the mixture of amino acids serine, methionine and glycine (1 mM each) by the letters SMG. Para amino benzoic acid represented by the letters PABA

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Materials and Methods

Bacterial Strains and Media. Bacterial strains are listed in Table 1. Minimal medium (M 63) as well as rich medium (LB) are described by Miller (1974). The minimal medium is routinely supplemented with 0.4% glucose and 5 μg/ml thiamine. When required, L-amino acids are added at a concentration of 50 μg/ml. All incubations are done at 37° C. Genetic symbols are according to Bachmann et al. (1977). All reagents used are of the highest purity commercially available.

Transductions. Generalised transductions were performed with P1 vir, according to the method described by Miller (1974). To transduce the *relA* mutations, we used the procedure described in Uzan and Danchin (1976), based on the selective inhibition of growth of the *relA* mutants by a mixture of serine, methionine and glycine (1 mM each). *hisT* derivatives were selected on minimal medium supplemented with isoleucine, methionine, uracile, adenine and thiamine and with 100 μg/ml each of aminotriazole and triazole-L-alanine and 200 μg/ml 4-azaleucine.

Table 1. Bacterial strains

Strain	Genotype	Source or reference
E. coli K 12		
KL 14	Hfr *thi-1 relA1* λ^-	Low via Springer
AB 1013	F′ *ilv R 269/thi-1 his-4 pro A2 lac y1 gal K 2 str-8 Δ(F′14) tfr-3 sup E 44*	Bachman
CP 78	F^- *thr leu arg his*	Fiil and Friesen
CP 79	$F^{-\prime}$ idem *rel A2*	1968 via Springer
FB 8	F^- prototrophic	Blasi
FB 8r	FB 8 *rel A1*	Arg^+ SMG-sensitive transductant from $P1(KL\ 14)_x$ FB 8 *arg A*
FB 105	FB 8 *his T 76*	Blasi
FB 8 rh	FB 8 *rel A1 his T 76*	$(AMT+TRA+AZL)^R$ derivative of FB 8r. (see text)
MI 366	F^- *thi-1 ilv O 603 mtl-1 mal A1 str-8 his-4 trpC lac 213 tsx-3*	Favre et al., 1976
MI 219h	HfrH *thi-1 ilv H 613 rel A1* λ^-	Iaccarino
MI 253 T	HfrH *thi-1 ilv H 612* or *613 ilv I 614 ara rel A1* λ^-	Iaccarino
MI 226a	MI 366 *ilv G* 605_{am}	Favre et al., 1976
1649	F^- *arg H trp E 9851 sup E 44 ? ilv A215*	Danchin, 1977
1636	F^- *arg H ilv A215 trp E 9851 sup E 44? rel A1*	Danchin, 1977
1637	1636 *ilv* A^+	P1 tranduction to Ile^+
1637o	1637 *ilv O 603*	Ile^+, Val^R transductants from 1636 × P1 (MI 366)
1637r	1637 *ilv R 269*	idem, except P1(AB 1013)
1637og	1637 *ilv O 603 ilv G* 605_{am}	Ile^+, Val^s transductants from 1636 × P1(MI 226a)
1638	1638 *ilv A 646*	*ilv A 646* from a cyclopentaneglycine resistant mutant. Iaccarino
E. coli B		
NF 541	*leu pyr B fuc*$^+$ *spo T rel*$^+$	Friesen
NF 542	NF 541 *rel A1*	

Threonine Deaminase Assay. The source of enzyme is a crude extract prepared as followed: An overnight culture of strain FB 8 grown in M 63 is diluted to 0.1 A_{650} units in 400 ml of the same medium. Cells are grown at 37° C with vigorous aeration and when turbidity reaches 0.5 A_{650} units the culture is rapidly chilled and cells are washed once with an equal volume of glucose free medium M 63. The cells are resuspended in 4 ml of 0.2 mM potassium phosphate buffer pH 7.5 containing 0.5 mM dithiothreitol, 1 mM EDTA and are sonicated for 2 min at 4° C with an MSE sonicator. The extract is centrifuged at 12,000 g for 15 min and the supernatant used as crude enzyme. Threonine deaminase activity is measured by following the ^{14}C-2-ketobutyrate formation from ^{14}C-L-threonine. The reaction mixture contains: 0.1 mM pyridoxal phosphate, 20 mM NH_4Cl, 100 mM Tris-HCl pH 8.0, 10 mM L-threonine, 0.5 μCi/ml of the ^{114}C-L-threonine (from New England Nuclear Corp.), 0.5 mM dithiothreitol and 100 μl/ml of crude enzyme. Each assay was performed in 1 ml, for 15 min at 37° C. The reaction is stopped by adding 0.5 ml of 2,4-dinitrophenylhydrazine (0.3% in 2N HCl) and tubes are then allowed to stand 10 min at room temperature. Then 2 ml of toluene are added and the tubes are vigorously shaken on a vortex. After a 5 min centrifugation to separate the organic and aquous phases, the radioactivity contained in 0.5 ml of the toluene supernatant is counted in a scintillation counter.

We usually found that about 1–2% of the radioactive input counts could be recovered in the toluene phase when the enzyme was omitted.

ppGpp and pppGpp Measurements. Measurements of the intracellular concentration of these two nucleoside phosphates is performed according to method described by Cashel (1969). The low phosphate buffer (1 mM KH_2PO_4) contains 50 μCi/ml of ^{32}Pi (CEA, France). Labeling was performed at a cell density of 0.18 A_{600} units.

Results

1. Addition of Excess One Carbon Amino Acids Leads to an Amino Acid Starvation

The addition of a mixture of the one carbon amino acids (SMG) in the millimolar range results in a rapid cessation of growth of either stringent or relaxed *E. coli* strains. For optimal manifestation of this phenomenon the cell density must be lower that 10^7 cells per ml. Under such conditions the growth of *relA* mutants is inhibited for at least 24 h (48 h in most cases) either in liquid or on solid media. The growth of stringent strains is only inhibited for a few hours (at 37° C).

As shown in Figure 1 a the incorporation of ^{14}C-phenylalanine is stopped very rapidly, at 37° C, in the couple of isogenic strains FB 8 (*relA*$^+$) and FB 8r (*relA1*) after addition of SMG. In stringent bacteria there is a parallele arrest of ^{3}H-uridine stable incorporation whereas this incorporation proceeds at a steady

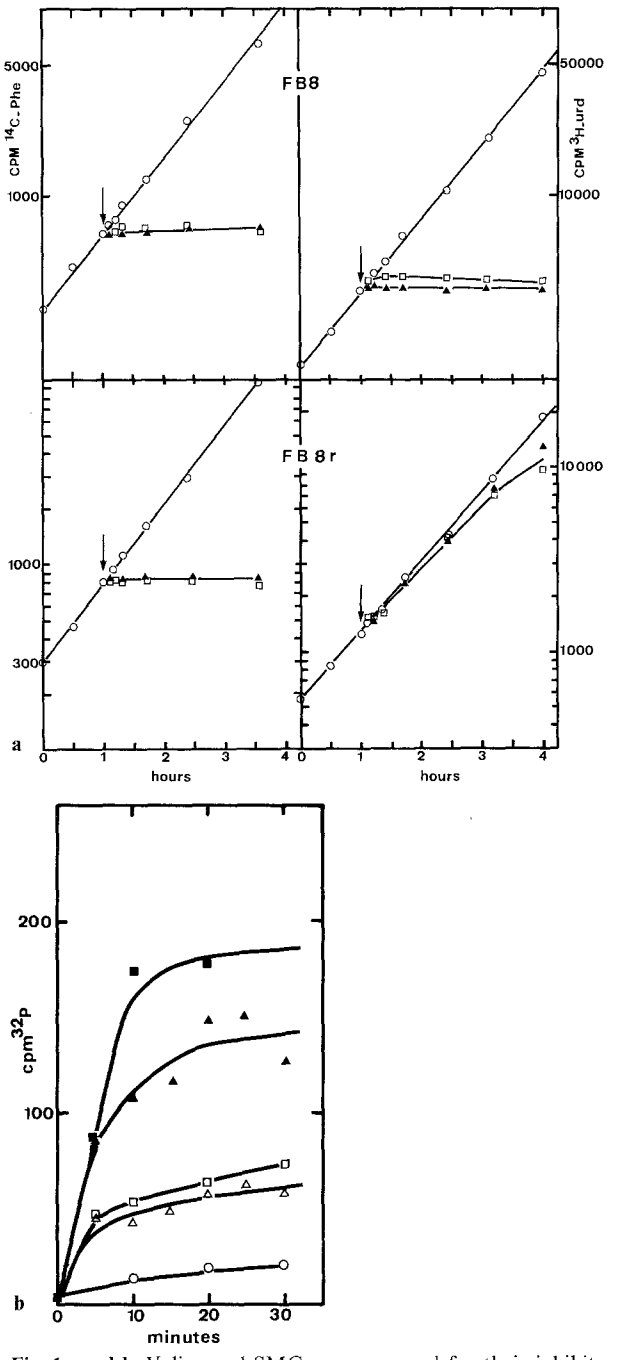

Fig. 1 a and b. Valine and SMG are compared for their inhibitory power on isogenic relaxed and stringent strains. **a** shows the incorporation of ^{14}C phenylalanine and ^{3}H uridine after addition of valine or SMG in strains FB 8 and FB 8r (2.5 µg/ml cold uridine and 0.4 µCi/ml of ^{3}H-uridine; 0.05 µCi/ml of ^{14}C-phenylalanine, 260 µCi/µmole). Labelling of the cultures was performed at a cell density of 10^{7}/ml. -▲-: + valine (200 µg/ml). -□-: + SMG (1 mM). **b** shows ppGpp and pppGpp levels after addition of valine or SMG to strains CP 78 (*relA*$^{+}$) and CP 79 (*relA2*).

ppGpp: −▲−: +SMG −■−: +valine
pppGpp: −△−: +SMG −□−: +valine
} strain CP 78

The pool of these nucleodides increase in the stringent bacteria but not in the relaxed mutants, as usually observed: −○− strain CP 79 and background incorporation (no inhibitors added)

rate in the relaxed mutant. Besides, as shown on Figure 1 b in the isogenic couple CP 78 (*relA*$^{+}$) and CP 79 (*relA2*) the levels of the nucleotides ppGpp and pppGpp increase in the stringent bacteria upon addition of SMG as well as upon addition of valine (which starves K 12 bacteria from isoleucine), and remain low in the relaxed mutant.

Thus the SMG effect presents all known features of an amino acid starvation.

2. Reversion of the Growth Inhibition Induced by SMG

We have tested the potential of individual amino acids to reverse the effect of SMG on growth of strain KL 14 (*relA1*). Among amino acids (except valine which inhibits *E. coli* K 12 growth) two were found to reverse SMG inhibition on plates. These were isoleucine and its precursor threonine. However a mixture of fifteen amino acids (all but SMG, isoleucine and valine) is unable to reverse inhibition although it contains threonine. We therefore tested couples of amino acids, containing threonine, on the growth of strain KL 14 in the presence of SMG and found that leucine alone is able to completely antagonize the reverting power of threonine. Moreover a mixture of fourteen amino acids (all but valine, isoleucine and leucine) is able to revert the inhibitory power of SMG whereas leucine restores inhibition (Table 2 a). Contrarywise isoleucine is fully able to relieve inhibition due to SMG either on plates or in liquid media. As shown on Figure 2, when added together with SMG it prevents the inhibition and when added some time after the inhibition has been initiated it restores normal growth after a lag time. The lag before growth is longer as the interval between SMG and isoleucine additions increases. Besides (Table 2 b) the relieving capacity of isoleucine is unaffected by the presence of leucine added together with SMG.

We were thus led to investigate the behaviour of the immediate precursors of isoleucine, in the presence or in the absence of leucine. Table 2 b shows clearly that all precursors tested, threonine, 2-ketobutyrate, 2-aceto 2-hydroxybutyrate, 2-keto 3-methylvalerate and isoleucine itself are able to revert the growth inhibition due to SMG. The relieving capacity of these compounds was then compared in liquid medium. Cultures of strain KL 14 (10^{5} cells per ml) are incubated at 37° C in synthetic medium containing SMG (1 mM each amino acid) and variable concentrations of isoleucine or its precursors. Turbidity is measured after a 24 h incubation period. It can be seen on Table 2 b that these precursors are all able to relieve the SMG inhibition. However these metabo-

Table 2. Reversal of SMG inhibition by branched chain amino acids and their precursors on strain KL 14

(a)

Added metabolite (1 mM each)	0	Σaa	Σaa −I−V	Σaa −I−V−L
Growth after 24 h	−	++	−	++

(b)

Added metabolite	T	2KB	2A2HB	2K3MV	I
Concentration required for 0.5 A_{650} after 24 h	150 μM	500 μM	~500 μM	~10 μM	9 μM
Growth in the presence of leucine	−	−	−	++	+

SMG, 1 mM each is added in all cases
Σaa: mixture of all amino acids except SMG. I: isoleucine, L: leucine, V: valine, T: threonine, 2 KB: 2-ketobutyrate, 2 A 2 HB: 2-aceto 2 hydroxybutyrate, 2 K 3 MV: 2-keto 3 methylvalerate
− No colonies after 24 h, + colonies of the same size as the control, ++ bigger colonies than observed on the control. See the text for other explanations

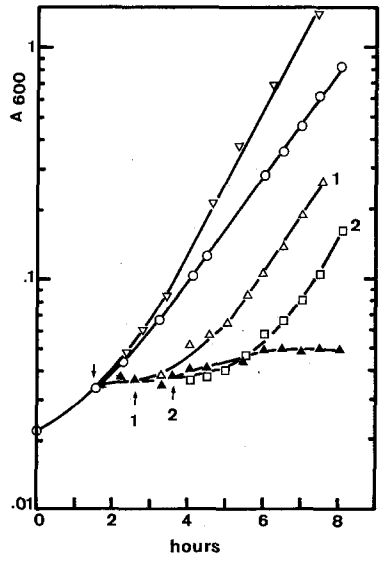

Fig. 2. Isoleucine reversion of the SMG growth inhibition. Growth of KL 14 (*relA1*) in the presence of SMG or SMG + isoleucine is followed by measuring turbidity of the cultures at 600 nm. Arrows indicate the time of addition of isoleucine −○−: control. −▲−: +SMG (1 mM). −▽−: SMG + isoleucine (1 mM). −△−: addition of isoleucine after one hour inhibition of the growth (curve 1). −□−: same after two hours (curve 2)

lites behave in quite different ways: the first three act only in the millimolar range, and their action is completely antagonized by leucine whereas the latter two act in the micromolar range and are insensitive to leucine addition. This suggests either a strong difference in sensitivity between the expression of the first and the last enzymes in the *ilv* operons with respect to the serine effect and/or a direct influence of isoleucine or its immediate precursor 2-keto 3-methylvalerate in a serine mediated inhibitory process.

3. The Specificity of the Serine Effect

The chemical structure of serine is very similar to that of the amino acid threonine and, since threonine is the normal precursor of isoleucine, and obvious explanation for the serine induced isoleucine starvation could be a straightforward competitive inhibition of the first enzyme of the pathway, threonine deaminase. In fact, Raskó and Alföldi (1971) found a strong competitive inhibitory action of serine on biosynthetic threonine deaminase and they proposed that this would be the basis of the serine effect. However this result contradicts the earlier observations made by Changeux (1964) who compared competitive and non competitive inhibitors of threonine deaminase and found that serine itself was a very poor competitor of threonine in the deaminating reaction.

We therefore undertook a reexamination of the competition of serine with threonine on biosynthetic threonine deaminase. We measured the activity of this enzyme in a sonicated extract of strain FB 8, using a radiometric assay (which is much more sensitive and specific than the colorimetric assay). Table 3 shows that, with a concentration of 10 mM threonine, one needs ten fold more serine in order to get an inhibition of about 60% of the activity. Because of the discrepancy of our results with those of Raskó and Alföldi we performed further measurements following in all details their experimental procedure (in particular, we prepared the crude enzyme from an old stationary culture, instead of an exponentially growing one). We failed to reproduce their results (using different strains), i.e. serine does not significantly inhibit the threonine deaminase activity.

Besides, the fact that 2-ketobutyrate, the product of the deaminating reaction, is as poorly efficient as threonine in releaving inhibition by serine, suggests that threonine deaminase is not the site of serine inhibition.

A similar argument applies to isoleucine transaminase, coded by the *ilv E* gene (Guardiola, 1977) which could have been inhibited by serine. From Table 2 it is clear that this is not so because the substrate

Table 3. Inhibition of L-threonine deaminase (biosynthetic) by L-serine

	^{14}C-Cpm in 0.5 ml Toluene	% inhibition
None	15,796	
Ile (1 mM)	1,753	100
L-Ser 10 mM	14,098	12
L-Ser 30 mM	10,973	34.5
L-Ser 50 mM	10,302	40
L-Ser 100 mM	7,639	59
No enzyme	1,878	

As expected one observes that isoleucine is a powerful inhibitor of this allosteric enzyme. Conversely serine appears to be a very poor inhibitor. The experiment was performed as described in Materials and Methods on a sonicated extract of strain FB 8

of the deaminating reaction, 2-keto 3-methylvalerate, is as efficient as isoleucine in relieving the growth inhibition induced by SMG.

All the other enzymes: the three acetolactate synthetases (*ilv B, ilv H I, ilv G*-coded), the isomero-reductase (*ilv C*-coded) and the dehydrase (*ilv D*-coded) are required not only for the synthesis of isoleucine but also for that of valine and leucine. If, therefore, serine was involved in a direct inhibition of these enzymes one should observe not only a starvation in isoleucine after SMG inhibition, but also a starvation in valine and leucine. One should therefore emphasize the fact that leucine *enhances* the serine effect.

The isoleucine starvation triggered by SMG is followed in *relA* mutants by a lack of isoleucine neosynthesis. We were thus led to investigate the behaviour of the *ilv* genes at the level of their expression in relaxed strains.

4. Lack of Derepressibility of ilv Genes in a relA Background

Transient limitation of growth by analogs of amino acids in *E. coli* has been described since a long time. It has been shown that, in this case, resumption of growth occurrs through a mechanism causing to derepression of the relevant biosynthetic pathways (Moyed, 1960; Roth et al., 1966). More recently, *relA* mutants have been shown to exhibit increased sensitivity to some analogs of amino acids as compared to the stringent parent (Stephens et al., 1975). The usual explanation for this is that derepression of certain amino acid operons is impaired in these *relA* mutants.

With respect to the specific isoleucine starvation when excess one carbon amino acids are added to cultures of stringent or relaxed bacteria, one would like to know whether the serine effect is amplified in the *relA* mutant because of the lack of derepressibility of some *ilv* genes.

We reasoned that, if this was so, then one should find regulatory mutations in the *ilv* gene expression that would overcome, at least in appropriate media, the inhibitory action of SMG. In particular we expected that mutations leading to constitutivity of *ilv* operons might decrease the sensitivity of *relA* strains to SMG.

Because of their apparently distinct genetic localization two different *cis*-acting regulatory mutations have been studied. They lead both to valine resistance and to constitutivity of the *ilv EJGDA* cluster. Table 4 shows that the introduction of either *ilv O 603* (Favre et al., 1976) or *ilv R269* (Ramakrishnan and Adelberg, 1964; Cohen and Jones, 1976) abolishes SMG sensitivity when bacteria are streaked on M 63 minimal plates supplemented with SMG and glucose as the carbon source (strains 1637o and 1637r respectively).

The *ilv O 603* mutation has been shown not only to result in derepressed expression of the *ilv EJGDA* cluster, but also to reveal a new acetolactate synthetase activity (encoded by the *ilv G* gene) unsensitive to valine inhibition, resulting in the valine resistant phenotype (Favre et al., 1976). We therefore asked whether this new acetolactate synthetase activity could be involved in the process leading to decreased SMG sensitivity. We constructed a derivative of strain 1637o carrying an amber mutation in the *ilv G* gene, by transducing strain 1636 (*ilv A 215*) with P1 vir grown on strain MI226a (*ilv O603, ilv G605*), selecting nor Ile$^+$ transductants and scoring the valine sensitive clones. In order to assess that the *ilv G605* mutation is indeed present in the transductant, we verified that these clones become valine resistant after lysogenisation with the amber suppressor phage ϕ80psuIII$^+$. As shown on Table 4 we found that this strain becomes SMG sensitive again. We must point out that this result does not prove that the activity of the *ilv G* gene must be absent in order to promote SMG sensitivity (because several other explanations might be invoked, including a polar effect of the amber mutation on some known or unknown *ilv* gene) but it is consistent with an involvment of this activity.

An explanation of the serine effect could be that, after addition of SMG, a sudden increase of the free valine pool appears inside the cell which leads to a general inhibition of the total acetolactate synthetase activities with ensuing isoleucine starvation. This is probably not the case because we found only 25 SMG resistant clones among a series of 100 spontaneous valine resistant derivatives of strain FB 8r. Five among the SMG sensitive, valine resistant mutants were mapped and found to map near *leu*,

Table 4. SMG sensitivity of relaxed strains harboring various *ilv* mutations

Strains	Relevant genotype	Sensitivity to	
		valine	SMG
1642	*rel*$^+$ (=1649 *ilv A*$^+$)	S	R
1637	*rel A1*	S	S
1637o	*rel A1 ilv 0603*	R	R
1637og	*rel A1 ilv 0603 ilv G 605*	S	S
1637r	*rel A1 ilv R269*	R	R
1638	*rel A1 ilv A 646*	S	S
MI 219h	*rel A1 ilv H 613*	R	S
FB 8	*rel A*$^+$	S	R
FB 8r	*rel A1*	S	S
FB 8rh	*rel A1 his T 76*	S	R

SMG sensitivity is tested here by streaking bacteria on M63 plates supplemented with SMG (1 mM each) or valine (1 mM), the required metabolites and glucose as the carbon source. The plates are read after 24 h incubation at 37° C

and are probably located at *ilv HI*. Furthermore, the valine resistant strain MI 219h (*relA1*, *ilv H613*), which yields an *ilv HI*-coded acetolactate synthetase insensitive to valine, is still sensitive to SMG (Table 4). Finally, we observed that, although resistant to valine as the usual *E. coli B* strains, a *relA* mutant of *E. coli B* (strain NF 542) exhibits a clear sensitivity to SMG, compared to its *relA*$^+$ parent NF 541. We can thus conclude that if the *ilv G* gene product is responsible for the decrease in sensitivity to SMG, this is not due to the valine resistance of the activity of this enzyme.

Another mutation is known to affect the regulation of *ilv* genes expression. Due to this mutation, *his T*, the isomerisation of certain uridylic acid residues to pseudo uridylic acid in the anticodon loop of several t RNA's species does not occur (Singer et al., 1972; Bruni et al., 1977). *his T* mutants of *Salmonella typhimurium* or of *Escherichia coli* have been found to be partially derepressed in the histidine and the isoleucine-leucine-valine pathways, and to have lost their ability to be repressed by exogeneous corepressors (Bresalier et al., 1975; Brenchley and Williams, 1975; Bruni et al., 1977; Lawther and Hatfield, 1977). This strongly suggests that t RNA's exert a significant regulatory role on the expression of some amino acid operons.

We introduced a *his T* mutation into a *relA* background by transducing strain FB 8r with P1vir grown on strain FB 105 (*his T76*) and selecting clones resistant to a mixture of the amino acid analogs aminotriazole, triazolalanine and 4-azaleucine. The selection medium was also enriched with several metabolites (see Materials and Methods) in order to allow a faster growth of the *his T* derivative. The actual presence of the *his T* mutation in the growing colonies was checked by showing P1 cotransduction between the amino acid analogs resistance character and the *pur F* marker in an appropriate recipient (Bruni et al., 1977). As shown in Table 4 we found that the strain obtained, FB 8rh (*relA1*, *his T76*) is resistant to SMG. Besides, as we show in Table 5, strain FB 8rh differs from its parent FB 8r in that it can overcome the growth inhibition observed when one shifts an exponentially growing culture from a rich medium (LB) to several different minimal media.

Derepression occurs at the level of transcription in the *ilv* operons (Lo Schiavo et al., 1975). We therefore asked whether certain mutants of RNA polymerase could not revert the serine effect. We isolated 96 spontaneous rifampicin resistant mutants of strain KL 14 (*relA1*). Among these, five rendered the mutant resistant to SMG. One of them, KL 14 RG 1, was kept for further study. First of all the relaxed phenotype was shown to be identical to that of the parent (continuation of ^{3}H-uridine incorporation after addition of 300 µg/ml valine, data not shown). Second the mutation was transduced in strain CP 79 (*relA2*), selecting for the rifampicin resistant character (55% cotransduction with *arg H*). This created an SMG resistant phenotype in the relaxed strain CP 79. These results indicate that a transcription step is necessary for relief of SMG inhibition.

The fact that the derepression of the *ilv* genes is of primary importance in the relief of the inhibitory action to SMG, is further indicated by the fact that prerepression of the *ilv* genes (by growing cells in a synthetic medium supplemented with an excess of isoleucine, leucine and valine) prior to addition of SMG results in isoleucine *and* valine starvation in the *relA* mutant but not in its *relA*$^+$ parent (Table 6).

Finally we should like to emphasize that, with respect to expression of SMG resistance, a derepression of the *ilv* genes is not correlated with other process that leads to overproduction of isoleucine. We observe that a *relA1 ilv A646* double mutant (strain 1638) which contains an isoleucine insensitive threonine deaminase and which is able to cross-feed *ilv A* (Ile$^-$) strains (M. Iaccarino, unpublished results) is as sensitive to SMG as its *ilv A*$^+$ parent (Table 6).

5. Interferences Between the One Carbon Metabolism and the Branched-Chain Amino Acids Synthesis

We previously described that a *gly A* mutation partially reverses SMG inhibition (Uzan and Danchin, 1976). We also found that addition of methionine

Table 5. Capacity of forming colonies after downshift transfer. Influence of the *his T* gene

Strain	LB	Mm	Mm + Methionine	Mm + Leucine	Mm + SMG
FB 8 (wild type)	100	100	100	100	100
FB 8r (*rel A1*)	100	62	0	0	0
FB 8rh (*rel A1 his T 76*)	100	100	95	25	21

Exponentially growing cells of strains FB 8, FB 8r and FB 8rh, in a rich medium (LB) at 37° C are diluted out and plated on various minimal plates (M63) supplemented with the indicated metabolites (1 mM each) and glucose as the carbon supply. The absolute number of colonies was 300–400 per plate on LB medium. Colonies were counted after a 48 h incubation at 37°C and the relative amount (colonies on LB/colonies on M63) is given

Table 6. Effect of a *rel A* mutation on the ability to express prerepressed *ilv* biosynthetic enzymes

Strains	Mm	Mm +SMG	Mm + SMG + Ile	Mm + SMG + Ile + Val
FB 8 (wild type)	100	100	100	100
FB 8r (*rel A1*)	100	0	2	85

Overnight cultures of strains FB 8 and FB 8r, grown in minimal medium supplemented with isoleucine, leucine and valine (1 mM each) were diluted in the same medium. When the turbidity of the cultures reached 0.3 A_{650} units cells were diluted and plated on minimal medium supplemented as indicated. Colonies were counted after 48 hours of incubation at 37° C. Results are expressed as percent of the colony forming capacity on the unsupplemented minimal medium (about 300 colonies per plate)

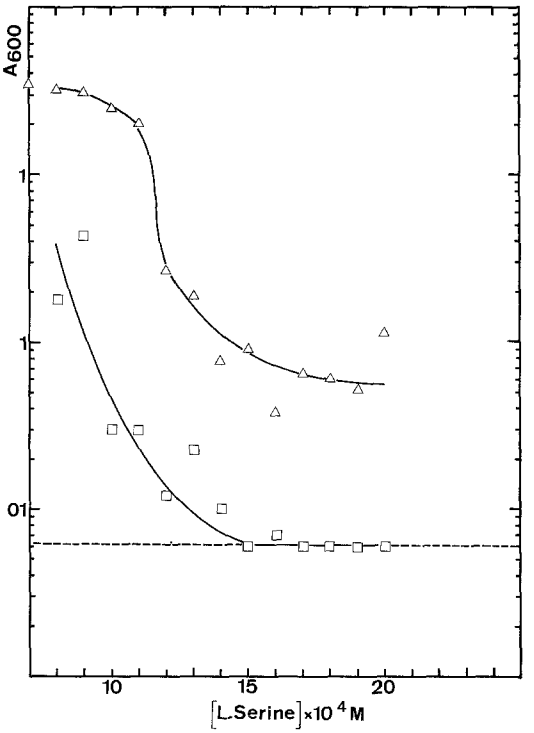

Fig. 3. p-Aminobenzoate reversion of the SMG growth inhibition. An overnight culture of FB 8r in minimal medium is diluted 10^4 fold in the same medium containing variable concentrations of serine, with and without 5×10^{-4} M of p-aminobenzoate (PABA). Turbidity of the culture is measured after 24 hours of incubation at 37°C. —□—: without PABA. —△—: +PABA. —▲—: control without serine. — —: absorption background

and glycine together with serine enhanced the inhibitory action of serine ten-fold. Since these amino acids are derived from the one carbon pool we looked for the effects of products interfering with the tetrahydrofolate synthesis.

Figure 3 shows that addition of 0.5 mM of paraaminobenzoic acid (PABA) shifts the inhibitory concentrations of L-serine on strain FB 8r to higher values. We found that no other vitamin is able to reverse the serine inhibitory action. One should note that these concentrations are very high as compared to the concentrations needed to support the growth of PABA requiring strains (about 0.01 μM, see for instance Baumstark et al., 1977). We also compared the influence of known inhibitors of tetrahydrofolate metabolism: sulfadiazine which inhibits incorporation of PABA into tetrahydrofolate and trimethoprim which prevents reduction of dihydrofolate to tetrahydrofolate. In order to have a comparison between $relA^-$ and $relA^+$ strains over a wide range of antibiotic concentration we used the impregnated disc technique (Kavanagh, 1975). 5 mm Whatman I paper discs were impregnated either with a 50 mg/ml solution of sulfadiazine or 5 mg/ml solution of trimethoprim and deposited on plates spread with a layer of CP 78 and CP 79: About 10^7 bacteria were used in order to ensure some growth of the relaxed strains when the plates are supplemented with one carbon metabolites. On unsupplemented plates, both strains are equally inhibited by both trimethoprim and sulfadiazine. However, when SMG and adenine (added in order to prevent a possible purine starvation) are included in the medium, a dramatic difference appears. $RelA^+$ and $relA^-$ bacteria are both equally inhibited by trimethoprim (no growth on a circle 40 mm in diameter). With sulfadiazine we observed confluent growth of the stringent bacteria at the contact of the disc but a circle of inhibition 54 mm in diameter in the case of the relaxed mutant.

Although as yet unexplained, these results are consistent with an involvment of tetrahydrofolate related metabolism in the serine effect. An interpretation

could be that some related metabolite interferes with the synthesis of isoleucine by a yet unknown mechanism, perhaps similar to the synthesis of pantoic acid which requires both the immediate precursor of valine, 2-ketoisovalerate, and 10-formyltetrahydrofolate (see Barman, 1974).

Discussion

In a previous article (Uzan and Danchin, 1976) we reported an extreme sensitivity of relaxed mutants of *E. coli* K 12 to serine in synthetic minimal media, as compared with the stringent parents. We use this property to separate *rel*$^-$ and *rel*$^+$ strains on Petri dishes, thus providing an easy way to perform genetic experiments with strains harboring *relA* mutations. We also showed that L-methionine and glycine greatly enhanced the inhibitory power of L-serine.

In this study, and in agreement with other authors (Cosloy and McFall, 1970, 1973; Raskó and Alföldi, 1971) we show that the basis of the serine inhibitory action is an isoleucine limitation. Whereas this inhibition is similar either on the stringent or on the relaxed strains in its *immediate* action it is rapidly overcome in the stringent bacteria and remains stable in the relaxed mutant. Thus the serine effect may be separated in two phases, first a sudden decrease in the available pool of isoleucine, and/or of charged tRNA^{ile}, common to both *relA*$^-$ and *relA*$^+$ strains, second a prevention of restoration of the normal pool in the relaxed mutants. We explain this latter SMG action according to the following mechanism. After the limitation has been established a process of derepression of the *ilv* genes tends to be initiated. However, in a *relA* background this process is very much impaired (whereas it promptly occurs in the wild type), so that a normal pool of isoleucine cannot be restored in the relaxed strains. In agreement with this proposition we found that mutants that contain constitutive mutations of the *ilv* biosynthetic pathway, such as *ilv O*, *ilv R* or *his T*, can overcome serine inhibition, under the experimental conditions we use. In fact we have found conditions where an exponentially growing culture of an *ilv O603 relA* mutant does not slow down its growth (as seen by measurements of turbidity) upon SMG addition, whereas the parent *ilv O*$^+$ *relA* stops growing under the same conditions.

Similar types of effects have been observed by M. Freundlich (1977), who has shown that derepression of threonine deaminase and acetolactate synthase is very much impaired in *relA* mutants when the cells are transfered from a rich broth to a glucose minimal medium.

The mechanism by which the *relA* gene influences the expression of some biosynthetic operons is not completely understood. Stephens et al. (1975) proposed that ppGpp could be a positive effector of the operons responsible for amino acid biosynthesis. In vivo as well as in vitro results obtained with the histidine (Stephens et al., 1975; Winkler et al., 1978), tryptophan (Morse and Morse, 1976), methionine (Sommerville and Ahmed, 1977), isoleucine-valine (Freundlich, 1977; Wild et al., 1977 and this work) operons are in agreement with this model. Contrarywise, Furano and Wittel (1977) showed, on growing cells, that ornithine carbamyl transferase, an enzyme responsible for arginine biosynthesis, is under the stringent control (see also Yang et al., 1974). Therefore, the influence of ppGpp on biosynthetic operons, if any in vivo, seems to be more complex than what could be infered from previous works.

One may remark here that there could exist a direct influence of serine (or one of its derivative) on the stringent coupling. Indeed serine biosynthesis is branched on the glycolytic pathway (from 3-phosphoglycerate). Since the carbon supply appears to control ppGpp synthesis and/or degradation (Gallant et al., 1976) one may wonder whether serine could not be involved in ppGpp metabolism.

It has to be emphasized that any situation that leads to an amino acid starvation in *relA*$^-$ cells results in considerable physiological distortions as compared to what happen in cells that have kept their stringent control (recently reviewed in Cashel 1975). The partial or total suppression by *his T* of the sensitivity of *relA*$^-$ mutants to down shift transfers, to SMG, and to some amino acid analogs (this was the method of selection of *his T relA* derivatives) strongly suggests that tRNA's play a major role in the enhancement of that sensitivity, possibly through their abnormal accumulation when *relA*$^-$ celles are submitted to particular stresses.

Two questions are posed by the SMG effect. One would like to know the reasons for the immediate fall in the isoleucine or ile tRNA^{ile} pools which occurs upon SMG addition. We should also like to understand the persistance of the effect in the *relA* mutants. As discussed above, this study has tried to answer the second question; besides it gives some hints about the answer to the first question.

Besides an impairment of the charging ability of the isoleucyl tRNA synthetase by SMG, three ways of decreasing the isoleucine pool inside a cell may be possible:

(1) inhibition of an enzyme in the *ilv* pathway specific for isoleucine;

(2) initiation of a permeation system which triggers excretion of isoleucine

(3) sudden enhancement of a metabolic pathway which consumes isoleucine.

We think that the results of the present study tend to rule out the first possibility, because the two isoleucine specific biosynthetic enzymes are clearly unsensitive to serine. However it could be that a metabolite, derived from serine, acts as an inhibitor of threonine deaminase. Another possibility, although unlikely, must be mentioned here. The fact that the presence of a functional *ilv G* gene is needed to overcome SMG inhibition may be interpreted as suggesting a specific inhibitory action of serine (or a derivative of serine) on the other acetolactate synthases than that encoded by *ilv G*. This would require a highly involved mechanisms, because this would mean that serine inhibits the 2-ketobutyrate reaction (which leads to isoleucine) but *not* the pyruvate reaction (which leads to valine) which both occur at the same catalytic site.

With respect to the second possibility (permeation) several results suggest that its contribution to the SMG effect might be significant. This is remarkable that all the amino acids which were shown to have a deleterious action on the growth of relaxed mutants in shift down conditions, namely phenylalanine, methionine, serine, leucine and isoleucine (Alföldi et al., 1963; Alföldi and Kerekes, 1964; Uzan and Danchin, 1976) share in common some of the transport systems responsible for branched chain amino acids permeability (Guardiola et al., 1974).

Moreover, Quay et al. (1977) have proposed that a component of the permeation machinery for branched chain amino acids (LIV I mediated) is involved in the excretion of isoleucine, when leucine (and also serine, albeit to a less isgnificant extent) is added. Such a contribution of isoleucine excretion would be more important in relaxed mutants because they lack, as we have shown, a full potential for derepression of the *ilv* operons. However the *synergistic* effect of leucine when added to serine (Table 3) is not explained right away by this permeation, because there should be competition at the same membrane site rather than addition.

A third hypothesis would be a metabolisation of isoleucine stimulated by the addition of excess SMG to exponentially growing cells. The model postulates that a metabolic pathway normally exists, that utilises a small fraction of the isoleucine pool (or of one of its precursors) and that SMG, through a mechanism which remains to be elucidated, greatly enhances the consumption of the branched-chain precursor of this pathway. There already exists a pathway leading to pantothenic acid from 2-ketoisovalerate, and utilising the 10-formyl-tetrahydrofolic acid. Furthermore, Fraser and Newman (1975) have shown that excess leucine (which increases serine inhibitory effect, see §2) triggers direct catabolism of threonine into the one carbon pool. Two systems are involved, an aldolase which catalyses the fission of threonine into glycine and acetaldehyde, and a pyridoxine-depending enzyme which enables the direct cleavage of glycine to one carbon units. We therefore feel that the conjecture assuming an interaction between serine derived and isoleucine derived substrates is worth considering in this frame. In this respect, knowledge of the relative concentrations of the isoleucine and valine precursors in different growth conditions (or in paticular mutants) should be of great interest.

We have devised a SMG supplemented medium where not only relaxed, but also stringent bacteria are unable to grow and mutants isolated under such conditions are the subject of our present studies, in order to challenge this last conjecture.

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