Molec. gen. Genet. 178, 155–164 (1980)

MGG

Serine Sensitivity of *Escherichia coli K 12*: Partial Characterization of a Serine Resistant Mutant that is Extremely Sensitive to 2-Ketobutyrate

Antoine Danchin and Liliane Dondon
Institut de Biologie Physico-Chimique, 13, rue Pierre et Marie Curie, F-75005 Paris, France

Summary. *E. coli* wild type bacteria display sensitivity towards serine. A selection medium is described which allows selection of serine resistant mutants. One such mutant is described which presents pleiotropic alterations: it exhibits a thermosensitive growth pattern, alteration in the metabolism of the pppGpp and ppGpp nucleotides, cAMP intracellular level alteration, extreme sensitivity to 2-ketobutyric acid and a defect in the phosphotransferases permeation system. A conjecture explaining these apparently unrelated defects supposes that serine metabolism interferes via phosphoenol pyruvate with a cytoplasmic control of membrane activity (the mutant would be defective in the coupling between membrane and the protein responsible for its cytoplasmic control) and that 2-ketobutyrate is an effector of this activity.

Introduction

Among the many pathways in the intricate metabolism of living cells one may define several general trends suggesting a hierarchical organisation in the production of some metabolites. A prominent feature in this respect is, on the one hand, the one-carbon metabolism, directly derived from glycolysis, through 3-phosphoglycerate and serine, and on the other hand, the aspartate and branched chain aminoacids metabolism, derived from the Krebs cycle. It has been shown that wild type *Escherichia coli* bacteria respond to addition of 1.5 mM serine in a minimum synthetic medium by an immediate cessation of growth for a extended period of time, related to the cell density and known or unknown alterations in their genotype (Uzan and Danchin, 1976). Additionally, input of a low amount of isoleucine (10 μM – 1 mM) was shown to completely overcome this action of serine (Uzan and Danchin, 1978). A series of experiments have suggested that many regulatory phenomena are related to the process of serine inhibition: cAMP mediated general regulation (Daniel and Danchin, 1979), expression of the branched chain aminoacids biosynthetic operons (Uzan and Danchin, 1978) and the stringent coupling of stable RNAs transcription to translation (Uzan and Danchin, 1976, 1978). It was therefore quite interesting to investigate whether mutants exhibiting an abnormal resistance to serine would point to a common target responsible for these pleiotropic effects. In this paper, we show some of the properties of a thermosensitive mutant exhibiting extreme resistance to serine. This mutant harbours a single mutation which, in addition to the thermosensitive (lethal) growth pattern displays the following main characteristics: a shift from permissive to non permissive temperature results in stop of stable RNAs synthesis, stable accumulation of ppGpp and continuation of protein synthesis; at high temperature the phosphoenol pyruvate mediated carbohydrate transport system is defective; growth of the mutant is sensitive to exogeneous threonine and extremely sensitive to 2-ketobutyrate addition; finally at any temperature the mutant is unable to propagate bacteriophage λ as a stable lysogen, whereas the lytic cycle of the phage is normal.

In this study, we show that a model involving a general control of the membrane active state in *E. coli* would account for all the properties of the mutant.

Materials and Methods

Bacterial Strains and Growth Media

The strains used in this study are listed in Table 1. We chose strain FB8 as a parental strain because it is entirely prototrophic.

Offprint requests to: A. Danchin

Abbreviations: smg: serine + methionine + glycine (addition to the growth medium); IPTG: isopropyl-β-D thiogalactoside (lactose operon inducer)

0026-8925/80/0178/0155/$02.00

Table 1. Bacterial strains

Strain	Genotype	Source or reference
FB 8	F⁻ prototrophic	F. Blasi
FB 8r	F⁻ *relA1*	Uzan and Danchin, 1978
SDT1	F⁻ *sbaA*	This work
SDT1 S	F⁻ *sbaA rpsL*	spontaneous Str^r
SDT1 R	F⁻ *sbaA rpoB*	spontaneous Rif^r
SDT1 N	F⁻ *sbaA nalA*	spontaneous Nal^r
SDT1 T	F⁻ *sbaA thyA*	spontaneous Trim^r(+Thy)
SDT1 S col. 17–28	SDT1 S harbouring plasmid n° 17–28 of the Clarke and Carbon bank	SDT1S crossed with JA200 n° 17–28 of Clarke and Carbon, 1977
SDT 132	F⁻ *sbaA argA*	SDT1T × P1DLA for Thy$^+$
SDT 1321	F⁻ *sbaA relA1*	SDT 132 × P1FB8r for Arg$^+$
SDT 152	F⁻ *ampA sbaA uxuA*	SDT1 × ϕ09+FAU for Amp^r
PP 15238	F⁻ *ampA valS*	SDT152 × C 38 for Uxu$^+$
ALS 1	F⁻ *argHI trpE9851 thyAts*	This work
DLA	F⁻ *proC purE trp lysA argA*	This work
For rapid mapping Hfr strains were used as in		K.B. Low, 1973 and A. Danchin, 1977
Other were:		
ϕ09+FAU	Hfr (9 min) *fol uxuA ampA metB*	This work
C 38	Hfr (14 min) *metC valS relA*	M. Springer
KL 14	Hfr (65 min) *thi-1 relA1*	see Uzan and Danchin, 1978
KL 14 V1	above + *ilv0603*	
KL 14 V4	above + *ilvR269*	
KL 14 RG1	above + *rpoBs*	
KL 25	Hfr (83 min) *supE42*	K.B. Low
Strains bearing episomes		
JC 1553 KLF 12	F′ (86 min *metB→pyrB*) *argG6 metB1 his1 leu6 recA1 mtl2 xyl7 malA1 gal6 lacY1 rpsL104 tonA2 tsx1 supE44*	B. Bachmann
Kl 132 KLF 17	F′ (96 min *purA→pyrB*) *thr1 leu6 thi-1 his1 pyrB31 pro27 thyA25 recA1 xyl 7 malA1 ara13 gal6 lacY1 rpsL9 tonA2*	B. Bachmann

The point of origin is given in parentheses on the *E. coli* modified map (Bachmann et al., 1976)

The usual media were the rich medium LB and the synthetic medium M63 (Miller, 1974). For the measurement of lysogeny of phage λ medium R (Miller, 1974), supplemented with 10 mM magnesium and 0.2% maltose (RMM medium) was used and 10^9 of the clear phages λ W30 and λ h80 W248 were plated together with the putative lysogenic bacteria. The clones harbouring integrated λ appeared as normal colonies whereas the clones harbouring non integrated λ appeared as small irregular translucent colonies.

After purification by streaking on LB plates, the former colonies maintain their aspect and were shown to be resistant to λ W30 and λ h80 W248, and sensitive to λvir whereas the latter acquired a normal aspect (i.e. grew bigger and lost their translucent appearance) and were shown to have reverted to sensitivity to phages W30 and W248, i.e. they had lost their host.

The selective medium (referred to as SD glycerol medium) contained the M63 salts together with serine, methionine, glycine, leucine (1 mM each), thiamine (10 μg/ml), sodium deoxycholate (0.5%) and glycerol (0.5%) as the carbon source.

Assays for Protein Synthesis and RNA Synthesis

^{14}C phenylalanine and ^{3}H uridine incorporation were measured as previously described (Danchin, 1977). When detailed protein analysis was required ^{35}S methionine was used as a label (850 Ci/mM) and the pulse chase procedure of Chao (1977) was used yielding one dimensional gel electrophoresis patterns where over than 60 proteins can be detected. β Galactosidase was measured after induction with 1 mM isopropyl-β-D thiogalactoside according to the standard procedure (Pardee et al., 1959). For pulse analysis a 5 min induction at the required temperature was performed and 100 μg rifampicin per ml was added to stop de novo RNA synthesis. Protein synthesis and assembly was then allowed to proceed at 28° C for at least 30 minutes before enzyme activity was measured.

ppGpp Synthesis and Turnover

ppGpp was measured according to the procedure of Cashel (1969) with slight modifications. The main alteration in the procedure were that the low phosphate synthetic medium contained 0.1 mM inorganic phosphate, and that EDTA (0.5 mM) was added to the samples before chromatography on PEI cellulose plates (polygram cel 300 PEI Machery Nagel and Co). The turnover was obtained by adding chloramphenicol (100 μg/ml) in the starved cultures and measuring the amount of residual ppGpp after different time intervals.

Phosphotransferase Permeation System

Radioactive α methylglucose (^{14}C; 184 mCi/mM, Amersham) was used as a non metabolized analog of glucose. Cultures grown to stationary phase were incubated in synthetic medium M63 with 0.4% glycerol as the carbon source, at different temperatures together with ^{14}C α methylglucose (1 μCi/ml). The permeation process of the glucose analog was followed by monitoring the radioactivity of 50 μl aliquots filtrated on Millipore nitrocellulose filters (0.45 μm pore size) and washed with 5 ml of a NaCl solution (9 g/l) at room temperature (20° C).

Rationale for the Selection of the Mutant

Bacteria have exquisite regulatory processes enabling them to adapt very efficiently to harsh changes in their environment. In particular,

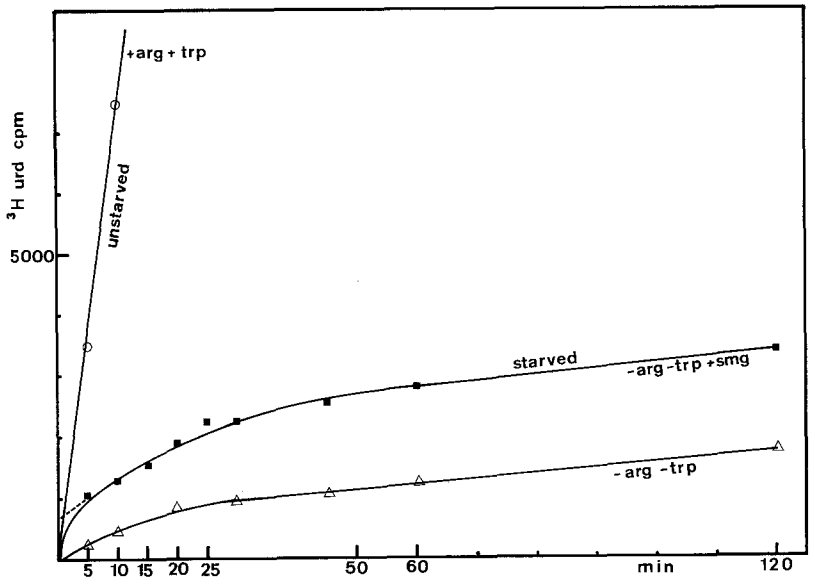


Fig. 1. Addition of one carbon amino acids (serine, methionine, glycine) results in delayed stop of stable RNAs synthesis in amino acid starved bacteria. A culture of strains ALS1, grown at 37° C to a density of 0.05 A_{550} in M63 medium supplemented with arginine, tryptophan (0.5 mM each) and four bases (adenine, guanine, cytosine and thymine, 0.1 mM each) is rapidly filtrated at 37° C on Millipore filters and resuspended either in the same medium –o–, or this medium devoid of arginine and tryptophane –■– or this latter medium supplemented with smg (1 mM each), –△–, together with (^{3}H) uridine. Samples are then precipitated with 10% trichloroacetic acid, filtrated on Whatman GF/C glass filters, and counted

the de novo synthesis of the machinery responsible for the general macromolecular synthesizing processes can be shown to be quickly induced or repressed, following shifts in culture media experienced by the bacteria. We therefore wished to test the conjecture that some feature of metabolism (namely a special unknown metabolite X_0) could not trigger, in a positive way, the de novo synthesis of this machinery (Danchin, 1979).

If such were the case, bacteria would be expected to be protected by a negative control in order to counteract the X_0 positive action, in case of non-adapted X_0 production. Therefore a way to find out the metabolic pathways leading to X_0 became obvious. It is known that starvation for almost any amino-acid leads to production of the ppGpp nucleotide which is parallel to turn off of a major part of the macromolecular synthesizing machinery.

It follows that a simple protection mechanism would be that X_0 derives from at least two *independent* amino-acid pathways so that any accidental saturation of a precursor would lead to both X_0 production and *starvation* for the other. This would then compensate for the accidental overproduction of X_0 because of ppGpp production.

aminoacid 1 → ? ⟶
aminoacid 2 → ? ⟶ X_0.

If such a conjecture is valid, there exists a very simple way to find out the pathways implied: a relaxed mutant, defective in correct ppGpp production, should be very sensitive to excess of the amino-acids necessary for X_0 production. Indeed it has been known for a long time that serine-derived amino-acids on the one hand, and the branched chain amino-acids on the other hand, strongly affect the growth properties of *relA* strains (Alföldi and Kerekes, 1968). More precisely an excess of serine stops the growth of these strains, and this effect is relieved by isoleucine (Uzan and Danchin, 1978). Conversely, an excess of threonine (20 mM) or isoleucine is also inhibitory, and is relieved by serine or methionine (data not shown).

These observations gave us a clue for the selection of mutants which would be affected in this hypothetical X_0 metabolite. Indeed, if its function is as pleiotropic as assumed it is clear that a medium rich in serine can lead to X_0 production which could at least transiently overcome the stringent phenomenon (see Fig. 1). Then, assuming that bacteria might be induced to increase in size during this period, and probably (because of the intrinsic unbalanced nature of the medium) be slightly defective in their cell wall synthesis (Ishiguro and Ramey, 1978) we added to the growth medium sodium deoxycholate, a detergent usually well tolerated by the intestinal bacteria *E. coli*. Finally, in order to have a maximum effect, taking into account all our previous observations (Uzan and Danchin, 1976; Danchin, 1977), in particular, the influence of catabolite repression, we ended with a medium containing serine, methionine, glycine, leucine, sodium deoxycholate and glycerol as the carbon source. On such a medium wild type *E. coli* bacteria are *killed* at 37° C and only mutants can grow. We wish to emphasize here that this does not by any means prove the conjecture but only gives the rationale for our isolation of mutants!

Results and Discussion

Isolation of the Mutant

In order to devise a medium which would inhibit growth of wild type *E. coli* bacteria according to the scheme outlined above, we investigated the properties of various media containing together with serine, different salt concentrations, carbon supply and other metabolites.

With respect to salt concentration medium M63 was found to be suitable for the experiment.

We know that a variety of metabolites interfere with the process of serine growth inhibition. Besides one-carbon metabolites, leucine has been shown to exert a synergistic influence on this phenomenon. Table 2 shows the borders of this effect of leucine, when we use mutants known to counteract serine inhibition such as *ilvR*, leading to derepression of the *ilvEDA* operon, or the *rpoB* allele described in a previous work (Uzan and Danchin, 1978). In addition, we

Table 2. Metabolic growth inhibition of *relA*$^+$ and *relA*$^-$ strains

	SMG		SMGL		SMGLI	
	+ DOC	− DOC	+ DOC	− DOC	+ DOC	− DOC
FB8	+++	+++	++	+++	+++	+++
FB8r	−	−	−	−	+++	+++
KL 25	+++	+++	++	+++	+++	+++
KL 14	−	−	−	−	+++	+++
KL 14 V1	++	+++	−	+++	+++	+++
KL 14 V4	++	+++	−	+++	+++	+++
KL 14 RG1	−	+++	−	+	+++	+++

Bacteria, pregrown on unsupplemented glucose M63 medium are streaked on agar plates containing the same medium supplemented with serine, methionine, glycine (SMG), serine, methionine, glycine, leucine (SMGL) and serine, methionine, glycine, leucine, isoleucine (SMGLI), 1 mM each amino acid in the presence (+DOC) or the absence (−DOC) of 0.5% sodium deoxycholate. After 24 h at 37° C the colony forming capacity is measured by the diameter of isolated growing colonies: $+++ \geqq 1.5$ mm; 1.5 mm > $++ \geqq 1$ mm; 1 mm > + and − no visible colonies

show that sodium deoxycholate added to the growth medium enhances the inhibitory pattern. At this point it appears that most relaxed strains will be sensitive to a M63 synthetic medium supplemented with serine, methionine, glycine, leucine (1 mM each) and 0.5% sodium deoxycholate (SD medium) and glucose as the carbon source but that a *rel*$^+$ strain will still be resistant. In order to increase the inhibitory potential of the SD medium, we investigated the influence of the carbon source and found that when glycerol replaced glucose a prototrophic wild type strain could no longer grow on this SD medium (see also Daniel and Danchin, 1979).

Typically, 10^8 to 10^9 bacteria of wild type FB8 strain, grown on M63 glucose medium without mutagenesis were spread onto SD glycerol plates and incubated at 37° C. After 48 h, 10 to 100 colonies appeared. These were purified on the same medium and their behaviour tested by replica plating on various media at different temperatures. One mutant, SDT1, clearly thermosensitive at 42° C on M63 glucose plates, and at 44° C on LB plates, was kept for further studies and is described in this work (other mutants will be described elsewhere).

Genetics of the Mutant

In order to localize the genetic defect, the rapid mapping technique described by B. Low was used (1973). For this purpose, derivatives of SDT1 harbouring markers for counter selection were isolated: *rpsL* (streptomycin resistance), *nalA* (nalidixic acid resistance) and *rpoB* (rifampicin resistance).

In order to recall the metabolic properties of the mutation we call it *sbaA* for (*s*erine-*b*ranched chain *a*mino acids metabolic control). The rapid mapping showed that Hfr Ra2 (point or origin 86 min) and R5 (point of origin 4 min) yielded thermoresistant recombinants at 42° C but not Hfr KL209 (origin 90 min), or Hfr H (origin 96 min). This suggests that the *sbaA* mutation maps in the region 90 min to 96 min.

We then constructed an Hfr strain for interrupted mating with derivatives of the SDT1 mutant. The Hfr ϕ09+FAU (refer to Table 1 for strain information) harbours markers designed to localize the mutation: namely *folA* (0.5 min) *uxuA* (97 min) *ampA* (93 min) and *metB* (87 min). The latter mutation was used for counterselection. Strain SDT1 or SDT132 was crossed with ϕ09+FAU and bacteria were plated at regular time intervals on agar plates containing trimethoprim (4 μg/ml), ampicillin (25 μg/ml) and incubated at 37° C. In the same experiment, samples of bacteria were also plated on minimal agar plates at 44° C for the scoring of thermoresistant recombinants. This experiment showed that the mutation was located near *uxuA*, probably between *uxuA* and *ampA*, near the *valS* marker. Having constructed a *valS* strain thermosensitive at 37° (as similar to the parent FB8 as possible in order to have comparable genetic backgrounds) we isolated *valS*$^+$ recombinants from a P1 lysate grown on strain SDT1 and assayed for thermosensitivity at 42° C and SD resistance.

About 75% among the transductants were found to be both thermosensitive and resistant to SD medium and several were assayed for λ lysogeny: they behave as does SDT1. Another procedure was to compare the transduction yield on M63 glucose and an SD glycerol at 37° C because the latter medium should yield only transductants both harbouring *sbaA* and *valS*$^+$. In this case one finds a 50% transduction efficiency on SD medium as compared to M63 glucose. Thus *sbaA* cotransduces 50–75% with *valS*. Due to possible ambiguities in transduction experiments we cannot be sure on which side of *valS* is the mutation. However, using the transduction values recently published by Isono and Kitakawa (1978) we place our mutation to the left of *valS* because we observed that it does not cotransduce with *uxuA*. This places the mutation very near the new ribosomal protein coding region found by these authors.

In order to study dominance or recessivity of the mutation, we crossed strain SDT152 (*sbaA, uxuA*) with strains JC1553 KLF12 and KL132 KLF17 harbouring the F′ factors KLF17 and KLF12. This yielded only bacteria which were still thermosensitive, thus suggesting either that the thermosensitivity is dominant or that the F′ factor did not harbour the

wild type allele of the mutated gene. Since large F′ factors are rather unstable, this latter possibility must be taken into account. We therefore tested the whole bank of colE1 plasmids harbouring fragments of *E. coli* genome prepared by Clarke and Carbon for complementation of a *rpsL* derivative of SDT1.

Two plasmids were found to complement, plasmids n° 17–28 and n° 41–28. After crossing with F^+ strains harbouring both of them and counterselection of the plasmid donor with streptomycin, we recovered micro-colonies at 44° C which grew well at 42° C on M63 glucose medium. The weak growth at 44° C suggests that the *ts* phenotype of SDT1 has not been overcome completely.

The Col E1 plasmid generally yields about 20 copies per cell; when compared with 1–2 copies of the F′ episomes, the results presented above (no complementation by F′ episomes, weak complementation by Col E1 plasmids) suggest that there might be a gene dosage effect in the complementation of the *ts* mutation, with many copies of a wild type gene being necessary to overcome the defect. It is possible, however, that complementation might be extracistronic.

The Col E1 plasmids of the Clarke and Carbon bank are expected to contain one-to-ten *E. coli* genes. The complementation observed is therefore compatible with a single mutation in SDT1 if one assumes that the plasmids carry a wild type allele of *sbaA*. In order to substantiate this interpretation, we selected for thermoresistant revertants and P1 transductants from P1 grown on a wild type strain and tested their different phenotypes (see below). Transductants restore all wild type characteristics whereas revertants restore most of them. This is compatible with a single mutation, *eliminated* by transduction, and *suppressed* either by intragenic or extragenic suppressors in the revertants.

Phenotype of the Mutant

Macromolecular Syntheses: Stop of Stable RNA Synthesis at High Temperature. In order to characterize the defect of SDT1 mutant, we measured the incorporation of ^{3}H-uridine, ^{3}H-thymidine and ^{14}C phenylalanine after a shift from 33° C to 43° C. As shown on Fig. 2a, we find that protein synthesis goes on for at least two hours, whereas RNA synthesis and DNA synthesis stop after 5 and 30 min respectively. No such effect of the temperature is found in the parental strain. Such an experiment only allows us to evaluate the overall incorporation of radioactive label in the macromolecules, therefore we followed a pulse incorporation of ^{35}S-methionine into proteins. After electrophoresis of the whole cell content on 10% acrylamide SDS gels (Fig. 2b), one observes that, after the temperature shift up the whole pattern is, apart from slight differences, very similar in both strains, showing that bulk mRNA synthesis is normal in the mutant under conditions where overall RNA synthesis is drastically decreased.

ppGpp Turnover is Defective in the Mutant

The simplest interpretation of the results just presented is that some metabolite inhibits stable RNA accumulation in the mutant. This prompted us to measure the pools of pppGpp and ppGpp upon temperature shifts. As shown on Fig. 3a, a temperature shift up results in transient pppGpp and ppGpp synthesis in the wild type strain (Gallant et al., 1977); in the mutant, the initial phase of ppGpp synthesis is comparable to what is observed in its parent, however, after a peak value has been reached (7–10 min) the concentration of ppGpp decreases much more slowly than in the parental strain. In parallel, the pppGpp accumulation is similar to that observed in *spoT* mutants, i.e. a small burst of synthesis is immediately followed by almost complete suppression of synthesis (Fiil et al., 1977).

A further confirmation of this interpretation comes from the fact that chloramphenicol addition is followed by a decrease of ppGpp concentration somewhat slower in the mutant than in its parent ($t1/2 = 12s$ in the wild type and 18s in the mutant at 44° C).

When SDT1 mutant is submitted to starvation for isoleucine induced by addition of exogeneous valine (3 mM) the metabolism of ppGpp is drastically altered (Fig. 3b). Indeed, we observe not only an increase in ppGpp production but also a dramatic increase in pppGpp synthesis. Under such conditions, SDT1 behaves as the *gpp* mutants recently isolated and described by Somerville and Ahmed (1979). Besides, we find that the steady state production of ppGpp is lowered in the mutant as compared with its wild type parent. All these results taken together are consistent with the interpretation given by Somerville and Ahmed of a dual control of pppGpp degradation to ppGpp exerted both by a *gpp* product (locus mapped at 83 min) and the *spoT* gene product (locus mapped at 81 min). Although mapped at a different locus (96 min), the *sbaA* mutation behaves as if it were like *spoT*.

Lambda Lysogeny is Defective in the Mutant

When incubated with a lambda wild type phage, our SDT1 mutant exhibits a peculiar behaviour. The plating efficiency of the phage shows that the yield of

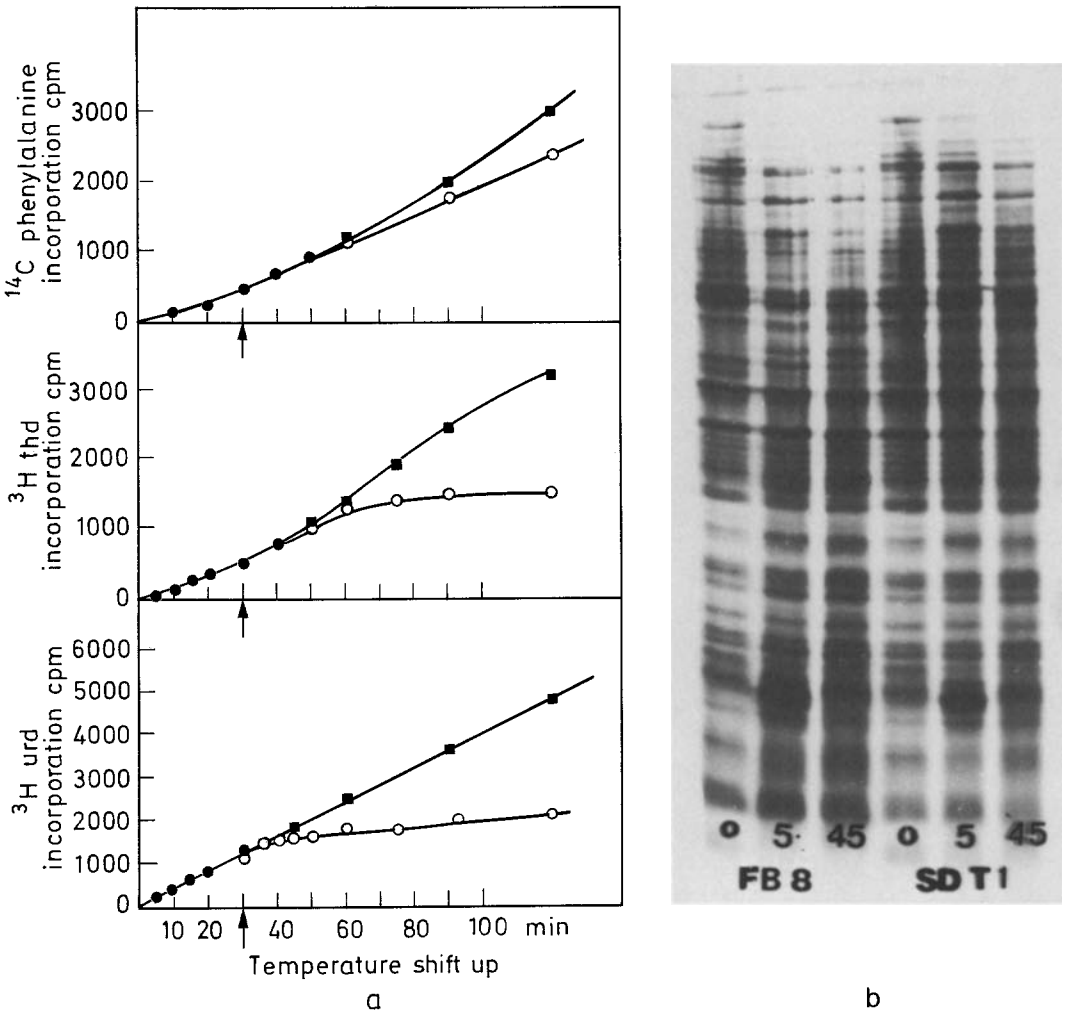


Fig. 2. a Macromolecular syntheses in strain SDT1 after a temperature shift from 33° C to 43° C. **b** 10% polyacrylamide gel electrophoresis of the whole bacterial proteins after a temperature shift (30° C to 43° C). Strain SDT and its wild type parent are compared after 0 min, 5 min and 45 min at 43° C. Proteins are labelled with ^{35}S methionine for 3 minutes and radioactivity is chased by addition of 1 mM methionine following the conditions of Chao (1977)

the lytic cycle is about 80% of that of the wild type, however the lysogenic cycle seems to be much altered. Under growth conditions where lysogeny is more than 90% efficient in the parental strain phage lambda fails to lysogenize the SDT1 mutant. This is true not only at high temperature (42° C), where the proportion of true lysogen to nonintegrated lysogens is 1/100, but also at 30° C where this proportion is about 20/100.

cAMP Accumulation is Impaired in the Mutant

Grodzicker et al. (1972) have shown that *inter alia* lambda lysogeny is sensitive to cAMP. More precisely when bacteria, defective in cAMP synthesis, were incubated with a lambda h80 phage these authors found that the bacteriophage failed to propagate as a stable lysogen. This prompted us to test influence of cAMP on our mutant. We therefore measured the lysogenization potential of the mutant in the presence and in the absence of cAMP. The experiment shows that addition of 4 mM exogeneous cAMP almost completely restores the normal lysogenization process (i.e. only 3 non lysogens among 100 colonies, as compared to 99 per cent in the absence of cAMP).

Therefore, SDT1 mutant behaves as if it were defective in cAMP intracellular accumulation. In order to substantiate this observation, we measured the capacity of de novo production of β-galactosidase at the non permissive temperature in the presence and absence of cAMP.

Figure 4 shows that in the parental strain the rate of β-galactosidase synthesis is increased, and after

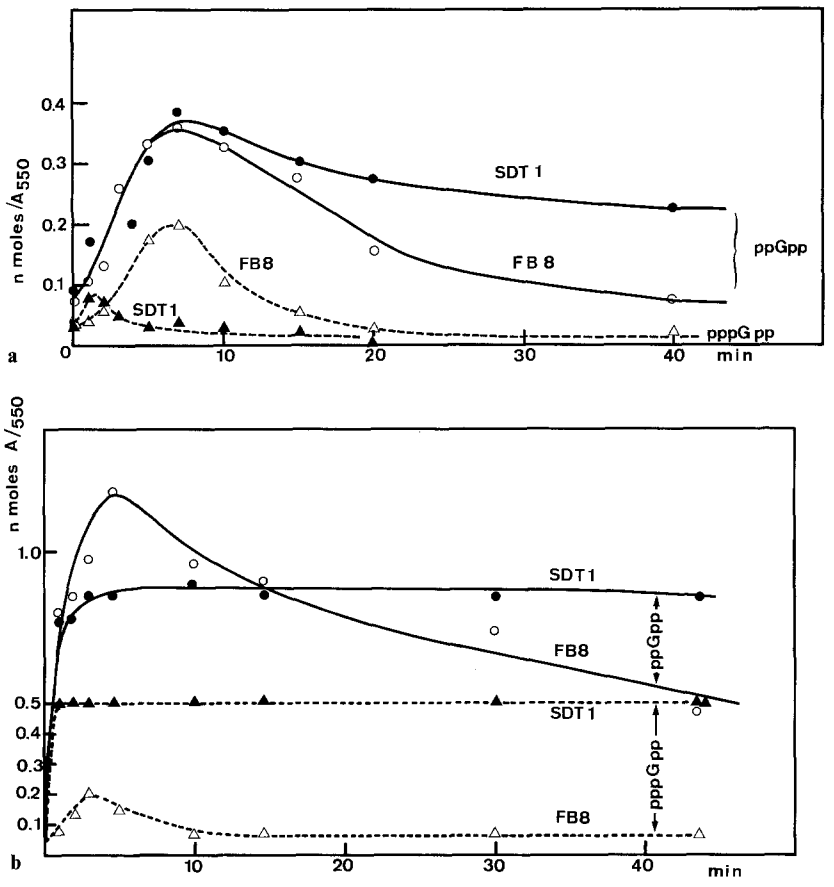


Fig. 3. a pppGpp and ppGpp accumulation in strains FB8 and SDT1, after a temperature shift up (30° to 44° C). ppGpp is only slowly degraded in strain SDT1. **b** same experiment after valine addition (3 mM) at 37° C. pppGpp is accumulated at a very high level in strain SDT1 (gpp like phenotype, see Somerville and Ahmed, 1979)

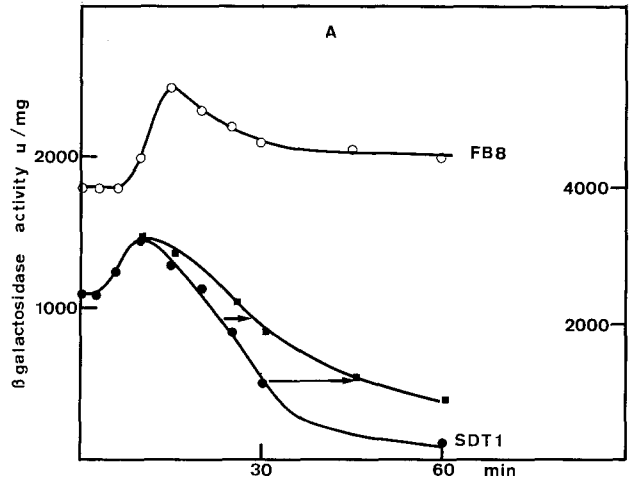


Fig. 4. β galactosidase rate of synthesis in SDT1 mutant at non permissive temperature. At various times after shift from 30° C to 44° C an exponential culture of strain SDT1 was incubated for 5 minutes in the presence of 1 mM IPTG, then 100 µg/ml rifampicin was added and the culture was transfered for 30 min at 28° C. β galactosidase activity was then assayed according to the procedure of Pardee et al. (1959). –○– parental strain; –●– *sbaA* mutant; –■– *sbaA* mutant in the presence of 1 mM cAMP (added at the time of temperature shift up): note that the ordinate is different in this latter case, for clarity of the comparison between inducibility in the presence and in the absence of cAMP.

a short overproduction, is maintained at a steady rate. In the mutant, on the contrary, 10 min after the temperature shift, the rate of β-galactosidase synthesis rapidly decreases. This is in contrast to the fact that overall protein synthesis is still very active (Fig. 2). When cAMP is added at the time of temperatureshift the decrease in the rate of β-galactosidase synthesis is strongly inhibited in the mutant. An alteration in cAMP intracellular level in the mutant is therefore consistent with these results.

The Phosphotransferase Carbohydrate Permeation System (pts) is Defective in the Mutant

It has been shown that the PEP dependent phosphorylating enzyme of the *pts* transport system (enzyme I), besides phosphorylating the "histidine protein" (Hpr) required for the carbohydrate transphosphorylation, regulates cAMP metabolism (Saier and Feucht, 1975). We therefore measured the global *pts* activity, revealed by the permeation of ^{14}C-α-CH_3-glucose in strains FB8, SDT1, SDT1S col. 17–28 at different temperatures. At 30° C there is only a slight

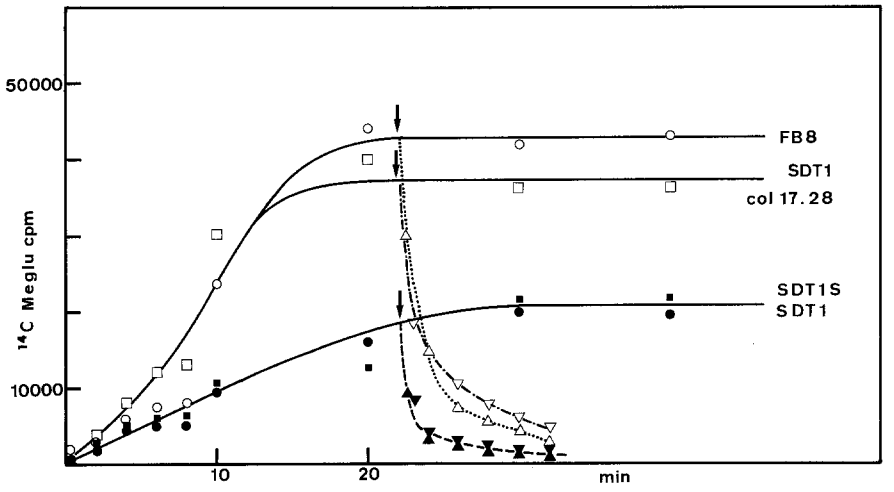


Fig. 5. αMe glucose accumulation in wild type, *sbaA* and *sbaA* complemented mutants at 42° C. Cells, grown at 30° C in M63 medium with 0.4% glycerol as their carbon source were filtered after incubation at 42° C in the presence of (^{14}C) αMe glucose, as described in Materials and Methods. –●– strain SDT1 (*sbaA*); –■– strain SDT1S (*sbaA rpsL*); –○– strain FB8 (wild type); –□– strain SDT1S col. 17–28 (strain SDT1S complemented with plasmid n° 17–28 of Clarke and Carbon *E. coli* bank). When the plateau level was reached non radioactive αMe glucose was added (1 mM) –▲– SDT1; –▼– SDT1S; –△– FB8; –▽– SDT1S col. 17–28

difference between these strains in their initial rate of permeation, the streptomycin resistant mutant having the less active permease. However it seems that the plateau level is lower in the mutant than in the wild type (data not shown). At 42° C the difference between mutants and wild type is enhanced (Fig. 5): not only does one find a lower plateau in the mutants but the initial rate of permeation is also significantly decreased. Besides, the behaviour of the complemented strain (harbouring plasmid n° 17–28) is intermediary between that of the wild type of its mutant parent.

This involvment of the *pts* system is further substantiated by the observation that SDT1 does not grow (at 42° C) on *pts* carbohydrate such as glucose, mannitol or sorbitol, either on liquid or solid media but grows for several generations when inoculated at a high density on the non *pts* carbohydrates maltose or lactose.

Among a large series of metabolites, several were found to restore some growth of the mutant at 42° C, namely serine, 2 ketoglutarate and isoleucine, conversely threonine, 2-ketobutyrate and 2-aminobutyrate were found to enhance the temperature sensitivity of the mutant (Table 3). At 37° C SDT1 is unable to grow when either 2-ketobutyrate or 2-aminobutyrate (1 mM) are added to the growth medium. This inhibition is relieved either by serine or by isoleucine. This latter result is, at first sight, paradoxical because threonine and 2-ketobutyrate are precursors of 2-keto-3-methylvalerate and isoleucine. However, it is well established that isoleucine is a feedback inhibitor of threonine deaminase so that our experiments point towards a specific influence of 2-ketobutyrate.

Pts activity was therefore assayed when 2-ketobutyrate or other keto acids are added to the growth medium. Figure 6 shows that strains FB8 and SDT1S col. 17–28 behave in a similar fashion and reveal that 2-ketobutyrate addition results in a *decrease* of the plateau level of ^{14}C CH_3 glucose in the bacteria. On the contrary strains SDT1 and SDT1S both show that upon addition of 2-ketobutyrate there is an *increase* of the permeation resulting in a new, higher, plateau. As controls pyruvate, and 2-keto 3-methylvalerate were used, the latter giving a small decrease in the plateau level in all four strains whereas pyruvate

Table 3. Effects of some metabolites on the colony forming potential of strain SDT1

Metabolites added (1 mm)	37° C	42° C		
		growth	mutants frequency	lethality
none	+++	–	10^{-7}	I
threonine	++	–	$<10^{-8}$	L
2-ketobutyrate	–	–	$<10^{-8}$	L
L2-amino butyrate	–	–	$<10^{-8}$	L
D2-amino butyrate	+++	–	10^{-7}	L
2-keto-3-methyl valerate	+++	+/–	10^{-5}	NL
isoleucine	+++	+/–	10^{-5}	NL
serine	+++	+/–	10^{-6}	NL
Smg	+++	+/–	10^{-5}	NL
2-ketoglutarate	+++	+/–	10^{-5}	NL
phosphoenol pyruvate	+++	–	10^{-6}	NL
cAMP	++	–	$<10^{-8}$	L

10^8 SDT1 bacteria, pregrown at 30° C on M63 medium supplemented with glucose where plated onto prewarmed M63 agar plates supplemented with glucose and thiamine (5 μg/ml) and various metabolites. Growth was observed after 48 h. Lethality was measured by the residual growth capacity remaining when the plates incubated at 42° C were further incubated at 37° C for 24 h: L, lethal, NL, non lethal, I, intermediate lethality. The thermoresistant mutation frequency was also evaluated from the number of colonies found to grow at 42° C

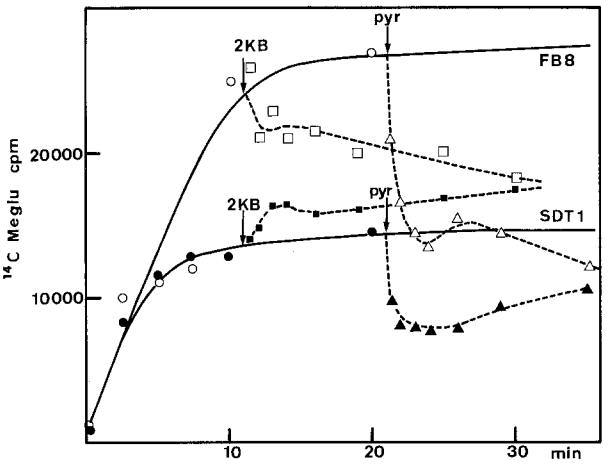
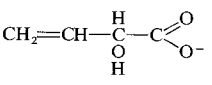


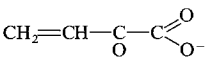
Fig. 6. αMe glucose accumulation at 37° C of a wild type and a *snaA* mutant: 2 ketobutyrate and pyruvate interferences with the accumulation process are compared. Experimental conditions are as in Fig. 5 except that 1 mM ketobutyrate or pyruvate are added when indicated. Strain FB8 –○– no addition, –□– 1 mM 2 ketobutyrate (2 KB) –△– 1 mM pyruvate (pyr). Strain SDT1 –●– no addition, –■– 1 mM 2 ketobutyrate –▲– 1 mM pyruvate

results in all cases, in a very complex inhibitory pattern, with a clear overshoot. Therfore it appears that 2-ketobutyrate exhibits a special behaviour with respect to the *pts* system, which is not solely due to its 2-keto acid structure.

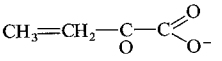
The structure and function of the *pts* system have been elucidated by Roseman and his collaborators (Postma and Roseman, 1976), and, recently, a picture somewhat more complex than initially assumed has emerged (Saier, 1977). In this picture several cytoplasmic and membrane components interact in a hierarchical fashion and cooperate for the transport of a phosphorylated form of the sugar permeant. Phosphoenol pyruvate, not ATP, is the phosphoryl donor used in this system and mutants of the various components have been isolated. Enzyme I mutants exhibit pleiotropic properties suggesting a function which is more general than plain phosphorylation of Hpr protein. The phosphorylated form would activate adenylate cyclase whereas the unphosphoryled form would inhibit this activity (Saier and Feucht, 1975). Therefore a correlation between *pts* and cAMP is already known. On the other hand, Walsh and Kaback, in 1973, made a puzzling discovery. When studying analogues of lactic acid they found that vinylglycolate,



is extremely toxic to bacteria due to its transformation into



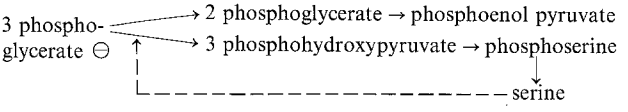
by the membrane lactate dehydrogenases and that this latter keto acid has a specific target in *E. coli, namely enzyme I of the phosphotransferase carbohydrate transport system* (Shaw et al., 1975). The resemblance to 2-ketobutyric acid



by this metabolite of vinylglycolate is striking and we interpret the inhibitory power of 2-ketobutyrate as being due to a specific interaction with the *pts* system, already somehow impaired in our mutant.

Conclusion

We thus propose the following scheme to explain the behaviour of our mutant. Glycolysis ends up, in almost equally equivalent quantities of serine and phosphoenol pyruvate:



and serine inhibits its own synthesis. Addition of a large excess of serine would therefore result in a transient burst of PEP. This excess would immediately act on the membrane dynamics and alter carbohydrate transport, ppGpp turnover and cAMP metabolism. Besides, by a yet unknown mechanism (which could be inhibition of an acetolactate synthetase activity, Uzan and Danchin, 1978) the excess would yield a transient overproduction of 2-ketobutyrate (with a parallel starvation of isoleucine). This metabolite would then counteract serine inhibition and the cell would resume normal growth after an appropriate lag phase. However serine addition would be drastically inhibitory for wild type strains in a detergent containing medium due to enhancement of membrane activity alteration. One would therfore expect to find, among mutants exhibiting high resistance toward serine, mutants defective in the coupling of PEP to its membrane related target. We think that this is the case of the mutation *sbaA*. This fact would explain all the alterations found such as (a) *spoT*-like ppGpp turnover (a large number of studies have suggested that glycolysis is somehow involved in the degradation of ppGpp: Winslow, 1971; Lazzarini et al., 1971; Hansen et al., 1975, eventually through a membrane

mediated process: De Boer et al., 1976), (b) cAMP accumulation defect and (c) *pts* defect together with extreme sensitivity to 2-ketobutyrate, because this metabolite is a negative effector of some of the functions of the *pts* enzyme I. Biochemical characterization of the SDT1 defect is now under current investigation and should bring appropriate answers to the questions raised by this hypothetical scheme. Finally we have also preliminary evidences about other mutants obtained with the same selection technique. Although they appear to map at different locations on the *E. coli* chromosome most of them share common properties: they have altered membrane activities, exhibit abnormal ppGpp turnover and abnormal regulation of the isoleucine – leucine – valine operons.

Acknowledgements. An experimental work is always a collective enterprise. We must therefore thank our colleagues for their constructive comments throughout this work: E. Brody, M. Springer and M. Uzan with reference to the genetical aspects, J. Daniel for discussion of the metabolic implications, C. Somerville for his enthusiastic critical interest, A. Ullmann for her sense of precision in experiments, conception and expression of the results, S. Busby for his critical comments on the manuscript and M. Grunberg-Manago for her kind hospitality.

This work benefited from grants of the Centre National de la Rechereche Scientifique (G.R. 18), Délégation Générale à la Recherche Scientifique et Technique (76.7.1198) and Institut National de la Santé et de la Recherche Médicale (ATP 52.77.84).

References

Alföldi, L., Kerekes, E.: Neutralization of the amino acid sensitivity of RC^{rel} *Escherichia coli.* Biochim. Biophys. Acta **91**, 155–157 (1964)

Bachmann, B.J., Low, K.B., Taylor, A.L.: Recalibrated linkage map of *Escherichia coli K12.* Bacteriol. Rev. **40**, 116–167 (1976)

Boer, H.A. De, Bakker, A.J., Weyer, W.J., Gruber, M.: The role of energy generating processes in the degradation of guanosine tetraphosphate, ppGpp, in *Escherichia coli.* Biochim. Biophys. Acta **432**, 361–368 (1976)

Cashel, M.: The control of ribonucleic acid synthesis in *Escherichia coli.* IV. Relevance of unusual phosphorylated compounds from aminoacid starved stringent strains. J. Biol. Chem. **244**, 3133–3141 (1969)

Chao, L.: Gene expression in stringent and relaxed strains of *Escherichia coli* during aminoacid starvation. Biochem. Biophys. Res. Commun. **77**, 42–49 (1977)

Clarke, L., Carbon, J.: A colony bank containing synthetic Col E1 hybrid plasmids representative of the entire *E. coli* genome. Cell **9**, 91–99 (1976)

Danchin, A.: A new technique for selection of sensitive and auxotrophic mutants of *E. coli:* isolation of a strain sensitive to an excess of one carbon metabolites. Mol. Gen. Genet. **150**, 293–299 (1977)

Danchin, A.: Is a metabolic control for the doubling of the macromolecule synthesizing machinery possible? Biochimie **61**, 45–50

Fiil, N.P., Willumsen, B.M., Friesen, J.D., Von Meyerburg, K.: Interaction of alleles of the *relA, relC* and *spoT* genes in *E. coli:* analysis of the interconversion of GTP, ppGpp and pppGpp. Mol. Gen. Genet. **150**, 87–101 (1977)

Gallant, J., Palmer, L., Pao, C.C.: Anamalous synthesis of ppGpp in growing cells. Cell **11**, 181–185 (1977)

Grodzicker, T., Arditti, R.R., Eisen, H.: Establishment of repression by lambdoid phage in catabolite achivator protein and adenylate cyclase mutants of *Escherichia coli.* Proc. Natl. Acad. Sci. U.S.A. **69**, 366–370 (1972)

Hansen, M.T., Pato, M.L., Molin, S., Fiil, N.P., Meyenburg, K. Von: Simple downshifts and resulting lack of correlation between ppGpp pool size and ribonucleic acid accumulation. J. Bacteriol. **122**, 585–591 (1975)

Ishiguro, E.E., Ramey, W.D.: Involvment of the relA gene product and feedback inhibition in the regulation of UDP-N-Acetylmuramyl peptide synthesis in *Escherichia coli.* J. Bacteriol. **135**, 766–774 (1978)

Isono, K., Kitakawa, M.: Cluster of ribosomal protein genes in *Escherichia coli* containing genes for proteins S6, S18 and L9. Proc. Natl. Acad. Sci. U.S.A. **75**, 6163–6167 (1978)

Lazzarini, R., Cashel, M., Gallant, J.: On the regulation of guanosine tetraphosphate levels in stringent and relaxed strains of *Escherichia coli.* J. Biol. Chem. **246**, 4381–4385 (1971)

Low, K.B.: Rapid mapping of conditional and auxotrophic mutations in *Escherichia coli* K12. J. Bacteriol. **113**, 798–812 (1973)

Miller, J.H.: Experiments in molecular genetics. New York: Cold Spring Harbour Laboratory 1974

Pardee, A.B., Jacob, F., Monod, J.: Genetic control and cytoplasmic expression of "inductibility" in the synthesis of β-galactosidase by *Escherichia coli.* J. Mol. Biol. **1**, 165–178 (1959)

Postma, P.W., Roseman, S.: The bacterial phosphoenolpyruvate sugar phosphotransferase system. Biochim. Biophys. Acta **457**, 213–257 (1976)

Rhoads, D.B., Epstein, W.: Energy coupling to net K^+ transport in *Escherichia coli.* J. Biol. Chem. **352**, 1394–1401 (1977)

Saier, M.H., Jr., Feucht,B.U.: Coordinate regulation of adenylate cyclase and carbohydrate permeases by the phosphoenol pyruvate: sugar phosphotransferase system in *Salmonella typhimurium.* J. Biol. Chem. **250**, 7078–7080 (1975)

Saier, M.H., Jr.: Bacterial phosphoenol pyruvate: sugar phosphotransferase systems: structural, functional and evolutionary interrelationships. Bacteriol. Rev. **41**, 856–871 (1977)

Shaw, L., Grau, F., Kaback, H.R., Hang, J.S., Walsh, C.T.: Vinylglycolate resistance in *Escherichia coli.* J. Bacteriol. **121**, 1047–1055 (1975)

Somerville, C.R., Ahmed, A.: Mutants of *Escherichia coli* defective in the degradation of guanosine 5′ triphosphate 3′ diphosphate (pppGpp). Mol. Gen. Genet. **169**, 315–323 (1979)

Walsh, C.T., Kaback, H.R.: Vinylglycolic acid: an inactivator of the phosphoenol pyruvate – phosphate transferase system in *Escherichia coli.* J. Biol. Chem. **248**, 5456–5462 (1973)

Winslow, R.M.: A consequence of the *rel* gene during a glucose to lactate downshift in *Escherichia coli.* J. Biol. Chem. **246**, 4872–4877 (1971)

Uzan, M., Danchin, A.: A rapid test for the $relA^-$ mutation in *E. coli.* Biochem. Biophys. Res. Commun. **69**, 751–758 (1976)

Uzan, M., Danchin, A.: Correlation between the serine sensitivity and the derepressibility of the *ilv* genes in *Escherichia coli* $relA^-$ mutants. Mol. Gen. Genet. **165**, 21–30 (1978)

Communicated by G. O'Donovan

Received June 1 / October 24, 1979