

THE OCCURRENCE OF SUPPRESSORS IN CAFFEINE-RESISTANT MUTANTS FROM *E. COLI* K12

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(Received March 3rd, 1969)

SUMMARY

Mutants resistant to lethal concentrations of caffeine (Caf^R) were derived from *Escherichia coli* K12. Caffeine resistance conferred on the mutants enhanced by a factor of 1.1–2.2 the resistance spontaneously shown by the wild-type strain.

Caffeine was not found to behave as a purine analogue in the biosynthetic pathway of purines. Neither cross-resistance to other purine analogues nor excretion or requirement in purine bases was observed.

Caffeine-resistant bacteria derived from UV repair deficient strains appeared to acquire an increased resistance to UV irradiation. Some of these mutants could partially repair the UV damage produced in the bacterial DNA whereas for phage DNA they were either fully or not at all able to perform host-cell reactivation of UV-irradiated phage λ .

In some Caf^R mutants there was a correlation between acquisition of caffeine resistance and suppression. About 80% of Caf^R mutants derived from *E. coli* K12 substrain 112–12 carried the amber suppressor *su-11*⁺. It is suggested that selection of Caf^R mutants might be a way of collecting bacteria with suppressors.

INTRODUCTION

In bacteria and bacteriophages, ultraviolet irradiation of wavelength 2537 Å is selectively absorbed by nucleic acids which are then damaged by UV-irradiation. Pyrimidine dimers are formed by UV light in the irradiated DNA (refs. 4, 34). The UV-damaged DNA can be repaired in the absence of visible light by enzymic processes which lead to the excision of pyrimidine dimers and to the restoration of the continuity of the DNA strands^{30,5}. These enzymes not only operate on bacterial DNA but also on double-stranded phage DNA such as T1 (ref. 28), λ (ref. 16), RF ϕ X 174 (ref. 17).

First LIEB²², then SAUERBIER²⁸ and METZGER²⁴, showed that caffeine inhibits the repair processes in UV-damaged DNA. The mechanism of caffeine's action is not yet established. It was recently proposed by ROULLAND-DUSSOIX²⁷ that caffeine could directly block the activity of repair enzymes. This hypothesis is based upon the fact

that caffeine inhibits the activity of a few nucleases *in vitro*. According to SHIMADA AND TAKAGI²⁹, thymine dimers are not excised from the UV-damaged DNA in the presence of caffeine.

From *Escherichia coli* K12, we isolated bacterial mutants more resistant than the original type to the toxic action of caffeine. Many of these mutants appeared also to be more resistant to the lethal effect of UV irradiation. The same result was independently found by GRIGG¹⁵ using *E. coli* B. If caffeine-resistant mutants were selected not from the wild-type strain but from host-cell reactivation deficient (Hcr⁻) strains, a partial restoration of the capacity to repair UV lesions was obtained. Bacterial survival as well as host-cell reactivation of UV-irradiated λ phage could be restored.

We have shown that restoration of the capacity to repair UV lesions in caffeine-resistant mutants derived from repair-deficient strains was very likely due to the correction by a suppressor of the original mutation responsible for the UV repair deficiency. We showed that in a bacterial strain carrying amber mutations, selection for caffeine resistance could lead to suppression of these amber mutations. Therefore, selection for caffeine resistance may result in indirect selection for suppressors and thus may permit us to determine whether a mutation is suppressible.

MATERIAL AND METHODS

Bacterial strains

The caffeine-resistant (Caf^R) bacterial mutants were derived from the *E. coli* K12 substrains shown in Table I. Strains CA 85, CA 5013, CA 161 and QD 5003 were used as control for suppression tests³² (Table I).

Selection of Caf^R mutants; level of caffeine resistance

Caffeine gradient preparation. The technique used to prepare caffeine gradients was derived from that of SZYBALSKI AND BRYSON³¹. 10-cm diameter petri dishes were slanted (10% declivity) and poured with 25 ml nutrient or L agar containing caffeine (usually 16 g/l). As soon as the bottom layer had hardened, dishes were laid horizontally and a second 25-ml nutrient or L agar layer without caffeine was poured. Such caffeine gradients appeared to be stable for at least 3 days.

Selection of Caf^R mutants. Samples containing 10⁹ cells of an overnight culture of the strain to be tested were plated with 3 ml soft agar on to caffeine gradient plates. Under such conditions cells grew on the side of the plate where caffeine was below the lethal concentration for the strain. Caf^R mutants appeared on a strip along the limit of normal cell growth.

Caffeine resistance. The caffeine resistance level was measured by either or both of the following techniques. (a) The resistance was estimated by the width of bacterial growth on gradient plates and expressed in cm along the concentration gradient. (b) Lethal caffeine concentrations were determined by using plain caffeine nutrient agar plates containing concentrations of caffeine ranging from 0.2 to 0.8%. Drops of 10³ bacteria were deposited on to these plates. After overnight incubation at 37° visible colonies grew only at permissible caffeine concentration levels. Strain GY 514, for instance, grew only in a concentration up to 3.5 g/l.

Caffeine gradient pattern. In order to establish the pattern of caffeine concen-

TABLE I
BACTERIAL STRAINS
Nomenclature is as proposed by DEMEREC *et al.*¹⁰ except that suppressors are indicated as in ref. 31. Moreover, cli means resistance to colicine I.

Strain No.	Origin	Sex	Radiation resistance	Nutritional requirements							Resistance to	Carbon source utilization	Sup-pressors	Obtained from		
				thr	leu	thi	thy	ura	cys	his						
K12 S	ref. 15	F ⁻	+	+	+	+	+	+	+	+	+	+	+	+	Sup I	RÖRSCH
K12 S hcr	Derived from K12 S ref. 15	F ⁻	hcr	+	+	+	+	+	+	+	+	+	+	+	Sup I	RÖRSCH
CR 34 thy	ref. 24	F ⁻	+	-	-	+	+	+	+	+	ton A	Y1	+	+	Sup II	JACOB
C 600	ref. 2	F ⁻	+	-	-	+	+	+	+	+	ton A	Y1	+	+	Sup II	WOLLMAN
GY 98	Spontaneous Clij ^R mutant from K12 S	F ⁻	+	+	+	+	+	+	+	+	cli-98	+	+	+	Sup I	This paper
GY 468	Spontaneous Clij ^R mutant from K12 S hcr ⁻	F ⁻	hcr	+	+	+	+	+	+	+	cli-468	+	+	+	Sup I	This paper
GY 514	Spontaneous Str ^R mutant from GY 98	F ⁻	+	+	+	+	+	+	+	+	cli-98 str-514	+	+	+	Sup I	This paper
GY 515	Spontaneous Str ^R mutant from GY 468	F ⁻	hcr	+	+	+	+	+	+	+	cli-468 str-515	+	+	+	Sup I	This paper
KMBL 49	Derived from CR 34 thy ref. 21	F ⁻	+	-	-	-	-	-	-	-	ton A	Y1	+	+	Sup II	RÖRSCH
GY 169	Spontaneous gal ⁻ from KMBL 90. Transduction gal ⁺ from C 600 by P1	F ⁻	uvr B 501	-	-	-	-	-	-	-	ton A	Y1	+	+	Sup II	This paper
KMBL 100	Derived from KMBL 49 ref. 21	F ⁻	uvr B 501	-	-	-	-	-	-	-	ton A	Y1	+	+	Sup II	RÖRSCH
I12-12	ref. 34	F ⁺	+	+	+	+	+	+	+	+	ton A	+	-	+	Sup II	WOLLMAN
CA 85	S. Brenner	HfrH	+	+	+	+	+	+	+	+	ton A	-	+	+	Sup III	THOMAS
CA 5013	S. Brenner	HfrH	+	+	+	+	+	+	+	+	-	+	+	+	Sup I	THOMAS
CA 161	S. Brenner	HfrH	+	+	+	+	+	+	+	+	-	+	+	+	Sup II	THOMAS
QD 5003	E. R. Signer	+	+	+	+	+	+	+	+	+	-	+	+	+	Sup III	THOMAS

tration gradient and to relate the 2 methods described above for the measurement of the caffeine resistance level, we examined the growth range of bacterial strains GY 514, GY 515 and MBL 49 on various plates containing increasing concentrations of caffeine in the bottom agar layer. In so doing we investigated the distribution of killing concentrations of caffeine at 3.5 and 4 g/l at the surface of various gradient plates (Fig. 1). This experiment showed that caffeine was not linearly distributed along the concentration axis. The caffeine concentration gradient was not constant.

The caffeine concentration at a given point on a plate cannot be derived from an arithmetical formula but can only be ascertained from the experimental curve establishing the gradient concentration pattern. Therefore, in the following we shall designate caffeine resistance levels by their experimental values given in cm.

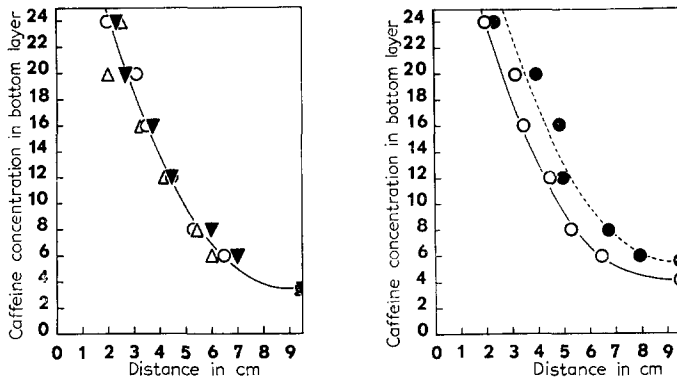


Fig. 1. Caffeine-concentration-gradient pattern. Caffeine-gradient plates were prepared with various caffeine concentrations ranging from 4 g/l to 24 g/l in the bottom agar layer as shown on the ordinate. Samples containing 10^9 cells of an overnight culture of the strain to be tested were plated with 3 ml soft agar on the gradient plates. On the abscissa are represented the width in cm of the bacterial lawn formed by strains MBL 49 (Δ), GY 514 (\blacktriangledown) and GY 515 (\circ). The general shape of a caffeine concentration gradient is represented without allowance for the fact that KMBL 49 is more sensitive than GY 514 and GY 515.

Fig. 2. Caffeine-resistance test on gradient plates. On gradient plates containing increasing caffeine concentrations in the bottom agar layer as plotted on the ordinate were plated 10^9 cells of strain GY 515 (\circ) and of its caffeine-resistant derivative GY 1211 (\bullet). The width of the bacterial lawn is plotted on the abscissa for both strains.

Test of amber suppressors

Amber suppressors were first tested by the ability of phage λ susR5 to grow on the Caf^R bacteria⁶. Later, a more extensive technique was used as described by VAN MONTAGU *et al.*³³ and THOMAS *et al.*³².

The following phages were used: λ susE43; λ susN7; λ susP3 (CAMPBELL⁶); λ susR216; λ susN213 (THOMAS³²); λ t57 (FUERST cited in ref. 32). Drops of various concentrations of λ sus phages were deposited on a layer of soft agar containing 10^9 bacteria per ml. Suppressors were evidenced by lytic spots.

Other techniques

Microbiological techniques. Bacteria were grown in well-aerated bubblers at 37° from overnight cultures diluted 1 in 20. Plating of phages and bacteria were carried out as described by ADAMS¹. Metabolic requirements were tested by the replica plating method²¹. Host-cell reactivation was performed with phage λ c72 (ref. 18).

UV irradiation. A General Electric germicidal lamp (15 W) was used. The exposure dose expressed in erg/mm² was measured by means of a LATARJET¹⁹ dosimeter. At a distance of 71 cm from the tube, the exposure dose was 10 erg/mm² per sec. The thickness of the bacterial cell suspension was less than 1 mm. Experiments were performed in a semi-dark room to avoid photoreactivation.

Media

(1) The following nutrient agar medium was particularly suited for the selection of caffeine-resistant mutants and the preparation of caffeine concentration gradients: peptone 8 g; bacto tryptone 5 g; NaCl 5 g; agar 15 g; distilled water 1 l. In most instances the addition of galactose 10 g/l to this medium very much improved the selection of mutants. Nevertheless, some caffeine-resistant mutants could only be isolated on L agar³.

(2) Ceria¹² mineral medium supplemented with glucose permitted the demonstration of caffeine resistance in liquid medium.

(3) NB (ref. 11) was used for bacterial cultures and phage λ stocks.

(4) M 63 (ref. 9) without glucose was used as buffer; supplemented with glucose, it was employed as a basic mineral medium to test auxotrophic strains.

(5) EMB medium²⁰ was used to test sugar fermentation.

(6) Caffeine was purchased from Touzart and Matignon (Paris), and Merck (Darmstadt).

RESULTS

Isolation of caffeine-resistant mutants

Isolation of bacterial mutants resistant to lethal concentrations of caffeine (Caf^R) was conducted as described in MATERIAL AND METHODS.

Caf^R mutants were first isolated from GY 514 and GY 515, derived respectively from K12s and K12s *hcr* (Table I) which is UV sensitive and host-cell reactivation deficient. Both strains are prototrophic and have a rapid growth rate.

Mutants from GY 514 and GY 515 generally appeared after a 2-day incubation and could easily be removed. However, Caf^R mutants from other bacterial strains showed up only after a few more days. Never could second-step mutants be isolated. A test for UV sensitivity showed that some of the Caf^R displayed an altered susceptibility to UV irradiation. Their phenotype will be described more extensively below. The above results made it desirable to test further whether Caf^R mutants could be derived from any bacterial strain and also whether a correlation might frequently be found between caffeine resistance and a modified UV sensitivity.

52 *E. coli* K12 substrains were studied. All but a few, such as GY 169, sported Caf^R mutants. Caf^R derivatives of 6 of the 52 substrains of *E. coli* K12 are described in this paper; the properties of other Caf^R mutants will be described elsewhere.

The range of caffeine resistance

Above a given caffeine concentration in the growth medium, wild-type bacteria can no longer divide. Such concentration is the upper tolerable caffeine concentration which corresponds to the background level of caffeine resistance for wild-type bacteria. In caffeine-resistant mutants the tolerable concentration can be more than

doubled. For the 52 substrains studied, the background levels of caffeine resistance could not be divided into definite classes nor could the range of resistance acquired by the Caf^R mutants. However, for the strains shown in Table II the maximal level of caffeine resistance acquired by the mutants was 1.5 times higher than the background level.

TABLE II

BACKGROUND LEVEL OF CAFFEINE RESISTANCE. CAFFEINE-RESISTANT MUTANTS: MUTATION FREQUENCY, RANGE OF RESISTANCE

Samples containing 10⁸ cells of the overnight cultures of the strains to be tested were poured with 3 ml soft agar on to gradient plates containing 16 g caffeine/l in the bottom agar layer. The widths of the bacterial lawns are expressed in cm as well as the range of increasing caffeine concentrations where the Caf^R colonies arise. Mutation frequency to caffeine resistance is the ratio of resistant colonies to the total number of plated cells.

Strains	Caffeine resistance: background level	Range of appearance of Caf ^R mutants	Mutation frequency
GY 514	5.0	5.0-5.6	5 · 10 ⁻⁶
GY 515	5.1	5.1-5.6	5 · 10 ⁻⁶
KMBL 49	2.6	2.6-4.1	10 ⁻⁵
GY 169	3.5-4.0	—	0
KMBL 100	3.0	3.0-4.0	10 ⁻⁵
I12-12	2.6	2.6-3.0	10 ⁻⁸

In Fig. 2, the extent of caffeine resistance displayed by GY 1211, one of the Caf^R derivatives of GY 515, is shown by plotting the range of bacterial growth over the plate *versus* the caffeine concentration in the bottom agar layer. The increment of the bacterial lawn is 1.2 cm; this value corresponds to a caffeine concentration increase of 1 g/l.

The spontaneous caffeine resistance level was variable with the strains studied, the range in which Caf^R mutants were found was narrow and the mutation frequency to caffeine resistance was rather high as shown in Table II.

Table III illustrates the variety of the extent of the caffeine resistance increase

TABLE III

CAFFEINE RESISTANCE LEVEL OF VARIOUS Caf^R MUTANTS

From gradient plates in the experiment illustrated in Table II, Caf^R mutant colonies were removed, purified once and then grown overnight at 37° in nutrient medium. The cultures were plated as described in Table II on gradient plates containing 16 g caffeine/l in the bottom agar layer. Measurement of caffeine resistance is expressed in cm.

Strain No.	Derived from	Width of lawn growth on gradient plates (cm)	Increased caffeine resistance over background (cm)
GY 1201	GY 514	6.2	1.2
GY 1202	GY 514	6.5	1.5
GY 1203	GY 514	6.2	1.2
GY 1204	GY 514	5.2	0.2
GY 1211	GY 515	6.5	1.4
GY 1214	GY 515	5.8	0.7
GY 1216	GY 515	5.2	0.1
GY 1231	GY 515	5.2	0.1
GY 1265	KMBL 49	6	3.4
GY 1268	KMBL 49	5.5	2.9
GY 1273	KMBL 49	5	2.4
GY 1278	KMBL 49	6	3.4
GY 1279	KMBL 49	5	2.4

of several Caf^R mutants as compared with the spontaneous level of the original strain from which they were derived.

Up to this point, caffeine resistance has been defined by tests performed on solid medium. However, it was difficult to demonstrate the extent of caffeine resistance in liquid medium until the Ceria synthetic medium was found to be particularly suitable. On inoculating this medium containing 2.5 g caffeine/l with 500 bacteria/ml, a total growth inhibition was observed for GY 515 bacteria whereas its derivative GY 1211 bacteria seemed to be far less affected. For the latter strain however the latent period and growth rate were respectively 3 and 2.5 times longer when caffeine was omitted.

Caffeine resistance and purine base metabolism

Cross-resistance to various purine base analogues was tested among Caf^R mutants derived from GY 514 and MBL 49.

Cross-resistance against 8-azaguanine (0.5 g and 5 g/l), 6-mercaptopurine (0.15 and 1.5 g/l) and 2,6-diaminopurine (0.325 and 3.25 g/l) was not found for the Caf^R mutants studied. Furthermore, 40 Caf^R mutants derived from strains GY 514 and GY 515 were studied to test whether some of them were regulatory mutants in the purine biosynthetic pathway. None of them excreted purine bases. Moreover, the Caf^R phenotype was never correlated with a purine requirement as verified for 32 Caf^R mutants derived from GY 514 and GY 515, 10 Caf^R mutants derived from MBL 49 and 15 Caf^R mutants derived from MBL 100.

UV susceptibility of Caf^R mutants

This study aimed at investigating the possible correlation between an altered UV susceptibility and the acquisition of caffeine resistance.

Preliminary tests showed that 20% of the derivatives of GY 514, 30% of GY 515 and 20% of KMBL 49 showed an altered radiosensitivity. These results were confirmed by investigating further the UV survival and the host-cell reactivation of a few Caf^R derivatives from GY 514 and GY 515 (Table IV). Mutants GY 1211 and GY 1214 displayed a partial recovery of the bacterial survival after UV irradiation as well as full restoration of the host-cell-activation property as shown in Fig. 3. However, survival of GY 1216 was much more radioresistant than GY 515 whereas host-cell reactivation was still lacking. Moreover strain GY 1231 was found to be more UV sensitive than its ancestor and still unable to perform host-cell reactivation.

Thus, starting from an Hcr⁻ strain we could find caffeine-resistant mutants which displayed a modified UV susceptibility. Bacterial survival was partially recovered but host-cell reactivation was restored either fully or not at all (Fig. 4).

Isolation of Caf^R mutants derived from GY 514 led to a surprising finding. Many of them were either more radiosensitive or more radioresistant than the original strain. However after rather long subcultivations they all lost the acquired phenotype. This is in contrast with GRIGG's results who isolated Caf^R mutants in *E. coli* B which appeared to be stable UV-sensitive bacteria.

Caffeine resistance and suppressors

The results described above indicate that a few Caf^R mutants displayed a

TABLE IV

UV SENSITIVITY OF SOME Caf^R MUTANT STRAINS: SURVIVAL AND HOST-CELL REACTIVATION OF UV IRRADIATED PHAGE λ

To test UV survival and host-cell reactivation of the isolated Caf^R strains experiments were carried out according to the legends of Figs. 4 and 5. In order to compare the various sensitivities of the mutants the 10% survival doses for colony formation and for host-cell reactivation were calculated from the curves. (1) After a few subcultivations these bacteria recovered their original phenotype. (2) Survival curves were rechecked after numerous subcultivations. In contrast, HCR properties did not change.

Mutants derived from	Caf ^R strains	Bacterial survival (2)		Host-cell reactivation
GY 514		1375	1375	3000
	GY 1201	200 (1)	1750	3000
	GY 1202	500 (1)	1250	2375
	GY 1203	400 (1)	1600	2650
	GY 1208	1200	1200	2425
	GY 1209	1300	1300	2280
	GY 1218	1225	1225	3000
GY 515		150	150	950
	GY 1211	800	875	2650
	GY 1214	625	625	2450
	GY 1216	425	525	1000
	GY 1231	75	75	950

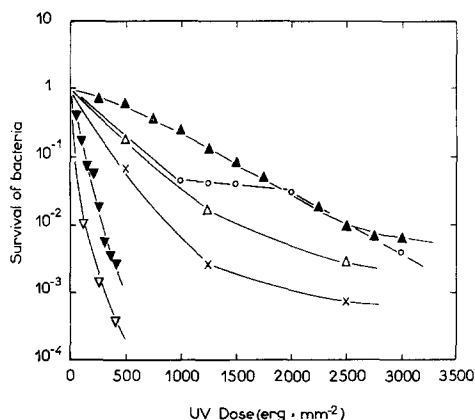


Fig. 3. UV survival of Caf^R derivatives of GY 515. Overnight cultures diluted 1 in 20 in fresh nutrient medium were grown at 37° for 5 h to reach 10⁹ cells/ml. Samples diluted a hundredfold in 0.01 M MgSO₄ were irradiated with increasing UV-doses shown on the abscissa, then diluted again, plated and incubated overnight at 37°. Survival of GY 514 (▲) and GY 515 (▼) and of the caffeine-resistant derivatives GY 1211 (○), GY 1214 (Δ), GY 1231 (▽), GY 1216 (×) are shown on the ordinate.

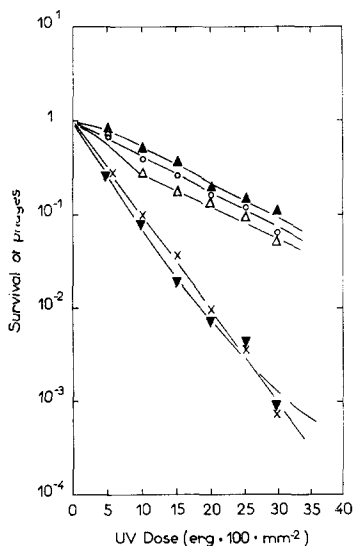


Fig. 4. Host-cell reactivation of phage λ by Caf^R derivatives of GY 515. Overnight cultures diluted 1 in 20 in fresh nutrient medium were grown at 37° to reach 10⁹ cells/ml. Phage λ C72 (ref. 18) at 10⁷ particles/ml was UV irradiated. 0.1-ml samples of phage dilutions were adsorbed on to 0.3 ml bacteria for 20 min at 37° and then plated and incubated overnight at 37°. On the ordinate are represented the phage survival on strains GY 515 (▼) GY 514 (▲) and caffeine-resistant derivatives GY 1211 (○), GY 1214 (Δ), GY 1216 (×). On the abscissa is shown the UV dose given to the strains.

phenotypic reversion of their UV sensitivity. This can be interpreted by assuming either a suppression or a reversion *in situ* of the *hcr*⁻ mutation.

(1) The latter hypothesis seems unlikely because of the extremely low probability of so frequently obtaining double mutants that are caffeine resistant as well as UV resistant. Moreover, the absence of parallelism between the recovery of bacterial survival after UV irradiation and the host-cell reactivation of irradiated phage λ is against a reversion of the *hcr*⁻ mutation.

(2) Our results can be explained by a suppressor type mutation which often produces a partial recovery of the missing property or function. To test this hypothesis we attempted to show that known suppressors would arise in mutants selected for caffeine resistance.

For this study, strain 112-12 was chosen for 2 reasons. Firstly, strains GY 514 and GY 515 used as basic strains were found to carry suppressor *su I*⁺ and KMBL 49 suppressor *su II*⁺. Secondly, in strain 112-12 it is known⁶ that introduction of suppressor *su II*⁺ permits the correction of the 2 amber mutations *cys*⁻ and *gal*⁻ as well as multiplication of conditional defective λ *sus* phages, whereas the *cys*⁻ mutation alone and a few conditional defective phages are corrected by suppressor *su I*⁺.

40 Caf^R mutants were derived from 112-12 on caffeine gradient plates with or without galactose. The mutation frequency was rather low: 10⁻⁸ of the plated bacteria. The caffeine resistance of the mutants over the spontaneous level of the original strain was very variable: it ranged from values close to the spontaneous level to twice as much.

When the 40 Caf^R mutants were checked for the presence of suppressors it was striking to see how the *cys*⁻ and *gal*⁻ mutations were corrected. As much as 28 of the Caf^R mutants became phenotypically Cys⁺ Gal⁺, 10 became Cys⁺ and 2 mutants still kept the original phenotype. The presence of suppressors in the mutants was further correlated with the finding that several mutants could grow phage λ *sus* R5. The type of suppressor involved was defined more precisely later on when the technique described by VAN MONTAGU *et al.*³³ was used. The Caf^R mutants could be divided into 5 classes as shown in Table V. More than 80% of the Caf^R mutants from 112-12 had acquired suppressor *su II*⁺ so that phenotypic reversion of *cys*⁻ and *gal*⁻ mutations was strongly correlated with the presence of this suppressor.

TABLE V

SUPPRESSION PATTERN IN Caf^R MUTANTS FROM 112-12

Caf^R mutants of strain 112-12 were tested for the acquisition of suppressors by the method of VAN MONTAGU *et al.*³³. The suppression pattern is deduced from the plating efficiency of the various conditional phages on strains C 600, CA 5013, CA 161, QD 5003, CA 85, which were used as controls. Requirements for histidine, cysteine and galactose fermentation were checked for each mutant by the replica plating method²¹.

Phenotype			Plating of phages λ <i>sus</i>							Suppressor pattern				Number of Caf ^R mutants of strain 112-12 in each class
<i>His</i>	<i>Cys</i>	<i>Gal</i>	N7	P3	R216	N213	E43	T57	+	<i>su</i> 112-12	<i>su I</i>	<i>su II</i>	<i>su III</i>	
-	+	+	+	+	+	+	+	-	+	+	-	+	-	27
-	+	-	+	+	+	+	+	-	+	+	-	+	-	5
-	+	-	-	-	+	+	-	-	+	+	-	-	-	5
-	+	+	-	-	+	+	-	-	+	+	-	-	-	1
-	-	-	-	-	+	+	-	-	+	+	-	-	-	2
-	-	-	-	-	+	+	-	-	+	+	-	-	-	Control 112-12

Some unexpected results were found; certain bacteria in which suppressor *su II*⁺ could be detected by growth of λ sus phages were still unable to ferment galactose. Moreover, some other *Cys*⁻ mutants seemed to carry no amber suppressor.

To check a possible fortuitous correlation between the *Caf*^R phenotype and the presence of *su II*⁺, *Cys*⁺ *Gal*⁻ revertants were first selected on minimal medium and tested for the presence of suppressor *su II*⁺; they were then tested for caffeine resistance. All the isolated bacteria were found to have an increased resistance to caffeine as compared with the original strain. Moreover, the caffeine-resistance levels paralleled those found in the mutants selected for caffeine resistance.

All the above results obtained with strain 112-12 were fully confirmed when caffeine-resistant mutants were derived from strain CA 85. The *lac*⁻ mutation was found to be corrected by suppressors such as *su II*.

In summary, the above data strongly indicate that there is a relationship between the caffeine-resistant phenotype and the acquisition of suppressors. These findings were confirmed by RÖRSCH (personal communication) who obtained phenotypic reversions of the *Hcr*⁻ character in *Caf*^R mutants isolated from various *Hcr*⁻ strains.

DISCUSSION

Caffeine and purine metabolic pathway

Caffeine is considered as an analogue of adenine and guanine, the purine bases found in DNA. Analogues of many biological compounds are known to be toxic agents, because they substitute for physiological metabolites. However, it was shown in the study of amino acid biosynthetic pathways that bacterial mutants resistant to the amino acid analogue could be isolated. Among analogue-resistant mutants, many regulatory mutants can be found^{7,8}: some produce enzymes at a very high level (derepressed mutants), others synthesize enzymes insensitive to feed-back inhibition (de-inhibited mutants).

Selection for bacterial mutants resistant to caffeine was expected to lead to the isolation of various types of mutants among which one could anticipate finding: (a) regulatory mutants in the purine biosynthetic pathway (derepressed or de-inhibited mutants); and (b) permeation mutants. This was not observed at all in *Caf*^R derivatives of GY 514 and GY 515. Our data provided no evidence that caffeine is a true metabolic analogue of purine bases.

Although cell permeation to caffeine was not specifically studied, some data (DELVAUX AND ERRERA, unpublished results) indicate that the uptake of radioactive caffeine is increased in the *Caf*^R mutants studied.

Caffeine resistance and UV repair

ROULLAND-DUSSOIX²⁷ demonstrated that *in vitro* a few nucleases were inhibited by caffeine. She put forward the hypothesis that the UV repair enzymes could be directly blocked by caffeine. As the isolation of amino acid analogue resistant bacteria led to the selection of regulatory mutants in the biosynthesis of amino acids we anticipated that similarly selection of *Caf*^R mutants would permit us to isolate regulatory mutants in the UV repair enzymic activity. Were this so, *Caf*^R mutants with a modified UV susceptibility should be easily collected. We obtained UV-resistant mutants which did not appear to display the expected properties of regulatory mutants.

Caf^R mutants GY 1211 and GY 1214 recovered only a partial UV repair activity, mostly exhibited as restoration of the host-cell reactivation capacity. One can explain such results by the hypothesis that only a small quantity of functional repair enzymes is present in these cells, just sufficient for the complete repair of phage λ DNA whose size is about 2% of the bacterial DNA. In contrast, UV survival of GY 1216 was higher than that of the original type but host-cell reactivation was not restored at all. In such event there is a net dissociation between the two repair capacities that are usually correlated: repair of bacterial DNA and repair of phage DNA. One can envisage that if recognition of the UV damage sustained by a specific DNA could play a role, the repair enzymes would have a better affinity towards bacterial DNA than phage DNA.

Caffeine resistance and suppression

The fact that selection for caffeine resistance in bacteria GY 515 devoid of dimer excision activity leads to a partial and variable restoration of the UV repair capacity suggests that the mutation *hcr*⁻ responsible for the UV repair deficiency is a suppressible one. Moreover, the hypothesis that suppressors could be selected by caffeine appears to be demonstrated in strain 112-12 whose Caf^R phenotype was accompanied in 80% of the mutants by the acquisition of suppressor *su II*⁺.

In strain 112-12, mutations *cys*⁻ and *gal*⁻ are usually corrected by suppressor *su II*⁺. This was also found for its Caf^R derivatives. However, in some Caf^R mutants the presence of suppressor *su II*⁺ was insufficient to correct the *gal*⁻ mutation. Moreover, Cys⁺ Gal⁻ derivatives were obtained which did not appear to carry any tested suppressor: one can argue that, in these bacteria, caffeine, by its mutagenic effect has reversed the original mutation. An alternative explanation is that suppressors other than amber could be selected by caffeine.

Caffeine and selection of suppressors

It is known that caffeine has a mutagenic action on bacteriophages²⁵ as well as on bacteria¹⁴. If one explained the acquisition of suppressors by a mutagenic action of caffeine, one would have to admit that the part of DNA coding for suppressor genes would be more specifically sensitive to the mutagenic action of caffeine. This hypothesis seems unlikely.

In its action on cells, caffeine probably produces reversible cellular alterations when removed after a short time. In a bacterial culture to which caffeine is added whose concentration is high enough to block cellular functions, a few cells only will survive that have escaped the inhibitory action of caffeine. Some of the bacteria that survive at caffeine lethal concentrations have acquired suppressors. One may suppose that caffeine acts either directly upon the genetic code or indirectly on the reading or translation of the genetic message.

A few experiments with radioactive caffeine show that 60-70% of the caffeine which goes into the bacterial cell is found with the cellular fraction where DNA is present (DELVAUX AND ERRERA, unpublished results). Yet the nature of the binding between caffeine and DNA is still unknown. One may suppose that caffeine could stick to the DNA and would thus permit the selection of Caf^R mutants able to read an altered code, so that it would allow the selection of suppressors. One cannot exclude, however, a physiological effect of caffeine on transfer RNA or ribosomes. Experiments to test these hypotheses are in progress.

ACKNOWLEDGEMENTS

This work has been supported by a Euratom fellowship to A. M. D. The method for testing suppressor genes was suggested by Dr. RENÉ THOMAS and was carried out in Dr. ERRERA's laboratory, under contract Euratom-ULB ABIB No. 007/61/10. We are grateful to Drs. ERRERA and THOMAS for their help and their interest in this work.

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