APPEARANCE OF DOUBLE MUTANTS IN AGED CULTURES OF *SALMONELLA TYPHIMURIUM* CYSTEINE-REQUIRING STRAINS[1]

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WHILE working with cysteine-requiring mutants of *Salmonella typhimurium*, we found that aged stocks of particular *cys* mutants predominantly contained bacteria carrying the *cys* mutation originally present plus an additional *cys* mutation. This paper reports investigations concerned with the origin, frequency, and some properties of these secondary *cys* mutations. Our data indicate that bacteria defective in gene *cysH*, blocked in the reduction of 3′-phosphoadenosine 5′-phosphosulfate, die during storage. There is a preferential survival of doubly mutant *cys* bacteria in which the deleterious flow of metabolites has been shut off. In addition, we detected in both aged wild-type and in aged *cys* cultures the presence of a relatively high content (about 10^{-4}) of bacteria spontaneously mutant for other loci in the chromosome.

MATERIALS AND METHODS

The auxotrophs used in this study arose spontaneously or were induced by mutagens in *Salmonella typhimurium* strains LT-2 and LT-7. Most of the strains have been described previously (MIZOBUCHI, DEMEREC and GILLESPIE 1962; DEMEREC, GILLESPIE and MIZOBUCHI, 1963; ITIKAWA and DEMEREC 1967). The Salmonella stocks are now available from Dr. KENNETH E. SANDERSON, Department of Biology, University of Calgary, Calgary, Alberta, Canada. Isolation of secondary mutations is described below.

The minimal medium contained KH_2PO_4, 4.5 g; K_2HPO_4, 10.5 g; $MgSO_4$, 0.5 g; $(NH_4)_2SO_4$, 1.0 g; sodium citrate·H_2O, 0.47 g; glucose, 5.0 g; sodium glutamate, 0.08 g; distilled water, 1000 ml. Each level of enrichment consisted of the addition of 0.2 g dehydrated Difco-nutrient broth plus 0.1 g NaCl per liter of medium. Single-, double-, and triple-enriched minimal plates were used, as specified for each experiment. Cysteine sulfinic acid (CSA) and sodium thiosulfate were added at final concentrations of 2×10^{-4} molar sulfur.

Working stocks of cysteine-requiring mutants of Salmonella were routinely maintained on Difco-nutrient agar to which 20 μg L-cysteine/ml had been added in order to compensate for the cysteine deficiency of commercial broth. Slants in screw-cap tubes were stored at about 4°C. They maintained viability for from 8 months to about 1½ years; transfers of mass inocula were made at intervals. The experiments reported in this paper concern observations made on such stocks; more recently (P. E. HARTMAN, personal communication) it has been found that Salmonella mutant stocks maintain their viability for periods of over 2 years if stored at room temperature in screw-cap tubes of Dorset egg medium (Fisher Scientific Co.).

Cultures were obtained from six to eight months aged nutrient agar slants by flooding each

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slant with 5 ml Difco-nutrient broth containing 20μg L-cysteine/ml and shaking overnight. Of this suspension, 0.5 ml was inoculated into 5 ml of fresh broth and grown for at least 8 hours at 37°C. The resulting culture was then used for transduction tests or for plating. Transduction tests were made by infecting with P22 phage at a multiplicity of five followed by plating on appropriately supplemented enriched plates. Wild-type P22 phage was used except where non-lysogenic derivatives were desired for detailed analysis. In the latter cases, infections were performed with the H-4 mutant phage (the *v1* phage of ZINDER 1958), and sensitive clones were obtained from isolated colonies restreaked on fresh medium.

Three methods were used to detect the presence of secondary mutations:

Method 1: Bacteria were infected with phage grown on wild-type bacteria and plated on supplemented agar. The agar contained an intermediate of cysteine biosynthesis that would not support the growth of the recipient bacteria but would support the growth of a secondary mutant blocked at an earlier step in the biosynthetic pathway. On medium containing a limiting amount of the intermediate, recombinants still carrying the secondary mutation, but now wild type for the original mutation would form small colonies. If the two mutations were linked in transduction tests, large (wild type) colonies also would be present. If no secondary mutation was present, all colonies would be large (wild type). Upon retesting, all but 10–20% of the small colonies proved to be cysteine mutants; the few remaining colonies were wild types that, through chance, had formed small colonies on the original test plates.

Method 2: Double mutants in which the secondary mutation led to a biochemical block *after* the block in the original mutant were detected by plating 100–200 viable bacteria from stock slants on specially supplemented plates. The plates were supplemented with an intermediate in amounts adequate to allow full growth (large colony formation) by the original mutant. In addition, the plates contained a limiting amount of cysteine. Under these conditions, double mutants were recognized as small colonies.

Method 3: In an effort to detect cases in which the nutritional requirement of the secondary mutant was identical to that of the original mutant, single colony isolates from revertible strains were cultured and subjected to stability tests. For this test, strains known to revert to the wild-type phenotype with diethyl sulfate (DES) were used. Cultures that did not show revertants (greatly decreased reversion frequency) were further examined. In transduction tests with phage grown on wild-type bacteria, no wild-type recombinants were expected when the two mutations in the double mutant were located in separate regions of the genome (i.e., located on separate transducing fragments). Double mutants in the *cysC* region (MIZOBUCHI *et al.* 1962; DEMEREC *et al.* 1963) could be recognized by altered transduction frequencies in tests with phage grown on other mutants located in this region. In cases where the original mutation was a deletion, stability tests were omitted and recombination was used as the sole criterion for determination of the presence of double mutants.

RESULTS

Recognition of the phenomenon: During the course of studies on the *cysC* region (DEMEREC 1960; DEMEREC *et al.* 1963; ITIKAWA and DEMEREC 1967) cultures of some strains appeared to have changed *en masse* in their recombination properties. This phenomenon has been repeatedly observed when old nutrient agar stocks, stored for a number of months at 4°C, were inoculated into broth and grown up for the tests. On one representative occasion, deletion mutant *cysHI536* was streaked on nutrient agar from an old slant and six single-colony isolates were tested as recipients in transduction experiments. Phage was grown on wild-type bacteria and on bacteria carrying *cysCD519*, a mutation cotransducible with *cysHI536* (Table 1). All sub-strains were phage-sensitive and required only cysteine for growth. Five of the six sub-lines of the strain carrying *cys-536* appeared to contain two closely linked mutations. Phage grown on wild-

TABLE 1

Transduction tests with progeny from single-colony isolates derived from an aged nutrient agar slant of cysHI536

Recipients	Donor (phage) *cysCD519*			Donor (phage) Wild type			Control (no phage)
	Large colonies	Small colonies	Percent recombinant	Large colonies	Small colonies	Percent recombinant	Number colonies
cys-536-1	0	850	0	101	176	36	0
cys-536-2	0	716	0	84	252	25	0
cys-536-3	0	1082	0	97	190	34	0
cys-536-4	0	970	0	88	170	34	0
cys-536-5	0	972	0	109	205	35	0
cys-536-6	686	302	69	369	2*	99.7	0

Three double-enriched minimal medium plates supplemented with 30 μg/ml CSA were used in each test. The *HI* mutant fails to grow on this medium, wild-type bacteria form large colonies, and bacteria mutant in genes *cysA*, *C*, *D* or *H* form small colonies (Table 2). DEMEREC *et al.* (1963) found about 53% recombination between deletion *cysCD519* and deletion *cysHI536*, comparable to the 69% in the single test shown below with progeny from colony *cys-536-6*. The other five derivatives appear to contain *cys* mutations in both the linked *CD* and *HI* regions.

* Two colonies composed of wild-type bacteria that appeared as small colonies on the original transduction plates. Most small colonies appearing on the other transduction test plates contain no wild-type bacteria.

type bacteria was able to effect formation of wild-type transductional clones; however, no wild-type clones were found in tests with phage grown on the deletion mutant *cysCD519*. This behavior is in contrast to that found earlier with the original *cysHI536* strain and, in these tests, with the sixth single-colony isolate of the strain carrying *cysHI536* (*cys-536-6* in Table 1). These results indicate that sub-lines 1 through 5 contained the original *cysHI536* mutation and, in addition, had acquired a second *cys* mutation in the *cysC* or *cysD* genes in the region covered by the *cysCD* deletion. Following observations such as this, a number of stocks available in the laboratory were examined for their content of secondary mutations.

Detection of mutants by method (1): Secondary mutants could be detected by using method (1) as noted above, i.e., by infecting with phage grown on wild-type bacteria and plating on suitably supplemented enriched plates. Bacteria with mutations in loci *cysI*, *J*, *HI*, and *HIJ* were plated on medium supplemented with CSA. Secondary mutants could be detected if present in the *cysA* cluster, complementation groups *Ba* and *Bc* of the *cysB* cluster, or in the *cysC*, *D*, or *H* loci of the *cysC* cluster (Table 2). Similarly, secondary mutants could be detected in *cysA* recipients plated on medium containing thiosulfate if present in the *cysC*, *D*, *G*, *H*, *I* or *J* loci.

Table 3 shows the results of transduction tests of various stock cultures with phage grown on wild-type bacteria. The recipients in Table 3 carry deletions that cover the *cysH* locus as well as the *cysI* or *IJ* loci. Nine of the eighteen stock cultures tested harbored secondary mutants in an overall frequency of greater than 1%. Similar results were obtained in preliminary experiments by K. MIZOBUCHI (personal communication), who also detected 15 secondary mu-

TABLE 2

Ability of mutants for various cys *loci to grow on several sources* (DREYFUSS and MONTY 1963; K. J. MONTY, *personal communication*)

Sulfur source	Wild type	Mutant classification*					
		C, D, H, CD	*HI, HIJ, G, I, J*	*Ba, Bc*	*Bb*	*Ea, Eb*	*Aa, Ab, Ac*
Sulfate	+	—	—	—	—	—	—
Cysteine sulfinic acid (CSA)	+	+	—	slow	—	—	+
Thiosulfate	+	+	+	—	—	—	—
L-cysteine	+	+	+	+	+	+	+

* Nomenclature for the *cys* loci is that of MIZOBUCHI *et al.* (1962) and DEMEREC *et al.* (1963). Multisite mutations involving more than one of several adjacent genes are designated by multiple letters for the defective genes; for example, a *cysCD* mutant contains a multisite mutation extending both into gene *cysC* and into gene *cysD*.

tants out of 2981 colonies tested from a different aged culture of *cysHI383* than that used here. The recipients in Table 4 carry deletions involving only one locus in the *cysC* cluster (i.e., *cysI68* and *cysJ538*), deletions such as *cysA20* and *cysA-533* that encompass the three *cysA* cistrons, and point mutations in the *cysI* and *J* loci. Only one secondary mutant was found among a total of 27,717 colonies tested from this group of cultures. This does not exceed the frequency of appearance of other auxotrophic markers in aged cultures (see below). The location of the lone secondary *cys* mutant from *cysJ262* was not determined. Additional tests with other aged cultures of *cysJ538* also failed to reveal the presence of secondary mutations (K. MIZOBUCHI, personal communication).

Detailed genetic analyses of non-lysogenic derivatives of 14 secondary mutants from the *HI* and *HIJ* cultures showed that each secondary mutation could be placed in the *cysC*, *cysD*, or *cysA* loci (Table 3). K MIZOBUCHI (personal communication) also has detected a *cysA* secondary mutation in a culture of *cysHIJ392*.

Detection of mutants by method (2): In method (2), the bacteria were plated on enriched medium supplemented with CSA (for *cysC, D, CD, H,* and *cysA* mutants) or thiosulfate (for *cysC, D, CD, H, HI, HIJ, I,* and *J* mutants). Double mutants were able to form small colonies at the expense of the broth enrichment while single mutants formed larger colonies at the expense of the CSA or thiosulfate supplements. Secondary mutants in the *cysI, J, Bb, E* and *G* loci or in the *cysA, B* and *E* loci could be detected on CSA and thiosulfate, respectively (Table 2). This experiment also served as a control for the detection of mutations at genes other than *cys* genes since new auxotrophs requiring a compound other than cysteine were recognizable as small colonies.

The data in Table 5 show that old cultures of Salmonella contain about 0.03% auxotrophic mutants. The auxotrophs often occur in small clones. Among these secondary mutants, leucine and methionine requirements predominate (cf. GROSS 1962). New *cys* mutations are rare in all of the cultures tested here, although the experiment was designed to detect double mutants involving a variety of *cys*

TABLE 3

Double mutants recovered from old slant cultures of deletion mutations covering the cysHI *or the* cysHIJ *loci*

Deletions	Loci involved	Transductant colonies observed	Small colonies harboring a secondary mutation			
			Total number	Number analyzed	Number different	Secondary mutant gene
cys-36	*HI*	841	8	1	1	*D*
536	*HI*	3928	98	3	2	*C, A*
cys-37	*HIJ*	342	0	..	..	..
66	*HIJ*	924	17	0	..	..
79	*HIJ*	600	0	..	..	..
305	*HIJ*	207	0	..	..	..
318	*HIJ*	910	0	..	..	..
352	*HIJ*	346	0	..	..	..
353	*HIJ*	2524	10	5	2	*C*
360	*HIJ*	1149	16	16	3	*C, C, D*
369	*HIJ*	615	0	..	..	..
383	*HIJ*	4931	0	..	..	..
388	*HIJ*	378	0	..	..	..
392	*HIJ*	3000	28	11	2	*C, D*
395	*HIJ*	201	16	0	..	..
399	*HIJ*	1349	35	3	2	*D, A*
431	*HIJ*	257	5	0	..	..
444	*HIJ*	79	0	..	..	..
Total colonies:		21,881	225	39	12	..

Double mutants were detected by infecting bacteria with phage grown on wild-type bacteria and plating on enriched plates supplemented with CSA, picking the small recombinant colonies, and testing these small colonies on minimal medium and on minimal medium supplemented with CSA. Column 4 shows the total number of colonies detected that grow on CSA but not on minimal medium. Column 6 indicates the *minimum* number of different secondary mutants; not all mutants were tested since it was not always possible to obtain non-lysogenic derivatives for use in genetic experiments. Also, some closely linked mutations of independent origin may have been classified together in some of our tests. The secondary mutations were assumed to be located in the *cysA* cluster if they failed to yield wild-type transductional clones in tests with *cysA20*, a deletion covering the three *cysA* cistons (MIZOBUCHI *et al.* 1962). The secondary mutations in the *cysC* and *cysD* loci were located by a series of tests similar to those shown in Table 1 for the *cys-536* derivatives. The last two columns list the minimum number and map locations of secondary mutations.

loci. Five *cysA* mutants were detected in *cysH398*, one *cysA*, *C*, or *D* mutant in *cysHIJ360*, five in *cysHIJ399* and two in *cysHIJ392*, and one *cysH*, *I*, or *J* mutant in *cysC1021*. While no stable secondary *cys* mutants were detected among the 4500 colonies of *cysH271* examined here, examination of another aged culture revealed the presence of a significant proportion of *cysA* double mutants (K. MIZOBUCHI, personal communication).

Detection of mutants by method (3): Thirty-nine single-colony isolates from each old culture of deletion mutants *cysCD519*, *cysH75*, *H364*, and *H398* were tested for their abilities to give rise to wild-type colonies upon infection with phage grown on *cysD313*, wild-type, and in some cases *csyCD519* bacteria. No presumptive double mutants were found from the former three strains, but the

TABLE 4

Double mutants recovered from old slant cultures of bacteria carrying deletions or point mutations in the cysI *or* J *loci or in the* cysA *cluster*

Strain	Locus or cluster	Number of colonies tested	Number of secondary mutations detected
Deletions:			
cys-68	*I*	1998	0
538	*J*	2609	0
20 (1)	*A*	1502	0
20 (2)	*A*	6600	0
20 (3)	*A*	6000	0
533	*A*	1584	0
Point mutations:			
cys-206	*I*	1095	0
230	*J*	1036	0
262	*J*	899	1
268	*I*	1043	0
292	*J*	925	0
299	*J*	678	0
307	*J*	561	0
316	*J*	554	0
494	*J*	973	0
Total:		27,717	1

Double mutants were detected by the method described in Table 3. Cultures of *cys-20* were tested from three different sources: (1) old nutrient broth, (2) old nutrient agar slant, and (3) old soft nutrient agar stab culture.

cysH398 culture consisted of 91% double mutants. The newly arisen mutations were located in *cysA* and in at least two different locations in *cysC*.

In tests of *cysH* point mutants, about 50–100 viable cells were plated on double-enriched plates supplemented with CSA. From each strain, 180 colonies were picked, separately cultured, and tested for their reversion responses by adding a drop of diethylsulfate to a triple-enriched plate containing about 2×10^8 bacteria. The colonies selected for study included all small colonies present on the plates as well as a sampling of larger colonies. The plates were incubated 96 hours at 37°C before scoring for reversions to prototrophy. Bacteria from cultures that did not revert were tested further. No secondary mutants were detected among the colonies examined from mutants in strain LT-2: *cysH38, 58, 241, 271, 354, 370, 534* and *551*, and from *cysH146* which is in strain LT-7 lacking a mutator gene. Three *cysH* mutants in strain LT-7 carrying a mutator gene (MIYAKE 1960) contained double mutants. The *cysH102* culture contained mutants for isoleucine-valine (1), tryptophan (3), and arginine (4); the *cysH112* culture contained lysine mutants (10); the *cysH163* culture contained mutants for pyrimidine (1), glycine (1), isoleucine-valine (3), purines (1), thiamine (1), and tryptophan (1). Since each of the clones found to be stable in reversion tests exhibited requirements for a second growth factor, we conclude that no secondary *cys* mutation was present in the 2160 colonies selected from plates containing

TABLE 5

Secondary mutations detected by method (2) in old slant cultures of various cys *mutants and in similar stocks of wild-type bacteria*

Strains	Number of strains tested	Total colonies tested	Number of different second mutants	
			cys	Others (classes found)
Deletions:				
cysHI	1	1,250	0	1 (unidentified)
HIJ	7	15,550	8	4 (purines, leucine)
H	4	13,450	5	0 .....
C or *CD*	2	4,450	1	2 (leucine, phenylalanine)
I or *J*	2	10,250	0	2 (methionine, phenylalanine)
A	2	10,400	0	3 (unidentified)
Point mutations:				
cysH	8	16,050	0	10 (leucine, methionine, lysine)
C or *D*	12	28,850	0	3 (leucine, methionine, unidentified)
I or *J*	9	23,050	0	2 (leucine)
Wild type:	10*	24,400	0	9 (unidentified)

The cultures were tested by plating about 200 viable cells on double enriched plates supplemented either with CSA or with thiosulfate. Small colonies were tested for their growth requirements.

* The 10 wild-type cultures were obtained from four different laboratories. Among the *HIJ* strains tested were *cys-66*, *353*, *362*, *388*, *392*, *395*, and *399*. Among the *H* strains tested was *cys-398*, from which the secondary mutations *cysC710*, *cysC* or *D711*, and *cys-712* were isolated.

29,628 colonies. Mutations in genes *B*, *E*, *G*, *I* and *J* would have led to small colony formation on the original plates containing CSA and would presumably have been readily detected and included among the 2160 colonies extensively tested.

Rarity of secondary mutations in growing cultures: The deletion strain *cysHIJ-392* (consult Table 3) was reisolated from single colonies. Three clones were cultured in nutrient broth containing 20 μg L-cysteine/ml and left at 37°C for four months. Samples were taken at monthly intervals and tested for the presence of double mutants by transduction with phage grown on wild-type bacteria. One secondary mutant was found after one month but none were found in the three later samples. A similar experiment was carried out by Y. NISHIOKA using deletion *cysHI536* (compare behavior with old cultures examined in Table 1 and Table 2). In this case, however, the culture was serially diluted in order to pass the bacteria through as many divisions as possible. The original inoculum contained about 200 bacteria in 50 ml broth. From this culture, once saturated, 0.5 ml was inoculated into 50 ml fresh broth. The procedure was repeated 11 times over a period of 2 months. Through the course of the experiment the bacteria passed through approximately 100 divisions. Bacteria from the final culture were infected with phage grown on wild-type bacteria and the mixture was plated on enriched plates containing CSA (Method 1). No secondary mutants were detected among 4000 recombinant colonies observed.

DISCUSSION

Double *cys* mutations are present in significant frequency in certain old cultures of *cysH* point mutants as well as in certain old cultures of *cysHI* and *cysHIJ* deletion mutants. An occasional aged culture is predominantly composed of double mutants. In each case tested, the secondary mutation occurred in one of the genes (*cysA*, *C* or *D*) involved in a step in cysteine biosynthesis preceding the reaction controlled by gene *cysH* (DREYFUSS and MONTY 1963). Secondary mutations are not found with high frequency in *cys* cultures defective in genes other than gene *cysH*. Gene *cysH* is involved in the control of the reduction of 3′-phosphoadenosine 5′-phosphosulfate or some similar metabolite (DREYFUSS and MONTY 1963). We presume that *cysH* mutants in old cultures accumulate a biosynthetic intermediate or some by-product that is deleterious to the preservation of the viability of the cell. Alternatively, a constant flow of metabolites through the early reactions may exert a drain on the supply of some critical compound, for example, ATP. In either case, double mutants in which the flow of metabolites has been lowered or eliminated would have a selective advantage.

Although no reconstruction experiments involving single and double mutants were performed, selection for secondary *cys* mutations does not appear to operate in fresh cultures. This is surmised by our failure to find a high frequency of double mutants in freshly transferred cultures and their rarity in many, but not all, aged cultures. In addition, we have observed that double mutants form dense uniform colonies that are readily distinguishable from the thin irregular colonies of the single mutant when bacteria are spread for isolated colonies on double-enriched plates supplemented with a limiting amount of L-cysteine. Thus, the operation of control mechanisms such as feedback inhibition and repression by cysteine in the medium may account for preservation of the single mutants in the absence of culture aging.

Phenomena of the type described here are of common occurrence. MURRAY (1965) mentions that *cys* mutants of Neurospora acquire secondary mutations in the biosynthetic pathway, but no evidence was presented on the pattern or mode of origin of the double mutants. Other cases of secondary mutations with selective advantage have been described for adenine mutants in Neurospora (MITCHELL and MITCHELL 1950), rhamnose mutants in Salmonella (ENGLESBERG and BARON 1959), galactose mutants in *E. coli* (KALCKAR *et al.* 1959; YARMOLINSKY *et al.* 1959; SUNDARARAJAN *et al.* 1962; FUKASAWA *et al.* 1963) and arabinose mutants of *E. coli* (ENGLESBERG *et al.* 1962).

The clustering of genes in Salmonella (DEMEREC 1964) is in part due to their organization into operons; however, one also finds cases where operons with related functions also are clustered (see AMES and MARTIN 1964). One force that could bring about clustering is a selection for double mutants. If the involved genes were juxtaposed on the chromosome, both mutations could be eliminated through a single genetic exchange involving a very restricted portion of the chromosome as is most often the case in bacteria. The *cysH* locus is linked by P22-mediated transduction, but slightly separated from, the *cysC* and *D* loci

(MIZOBUCHI *et al.* 1962; DEMEREC *et al.* 1963). The *cysA* locus is not cotransducible with *cysH*, but it is located near to *cysH* on the Salmonella chromosome (SANDERSON and DEMEREC 1965; ROTH, ANTÓN and HARTMAN 1966). Other cases resulting from interactions between single and double mutations of the type described by GROSS (1962) also might lead to propensities for clustering of genes not involving the same metabolic chain.

Old Salmonella cultures contain a variable but often high (about 10^{-4}) content of auxotrophic mutations other than *cys* mutations. The occurrence of spontaneous mutations in stationary phase populations of *E. coli* has been studied by RYAN and coworkers (see RYAN, NAKADA and SCHNEIDER 1961), and specific types of mutations have been found to occur in stored bacteriophage suspensions (DRAKE and MCGUIRE 1967). The secondary *cys* mutations found in our aged populations require further tests to establish their molecular nature.

DRS. PHILIP E. HARTMAN and KENNETH E. SANDERSON assisted in the preparation of this manuscript, based largely on a draft written in 1962 by the two senior authors.

SUMMARY

An unexpectedly high proportion (about 10^{-4}) of bacteria in old nutrient agar slant stocks of *Salmonella typhimurium* carry mutations at any one of a number of loci on the chromosome. These mutations are presumed to arise as spontaneous mutations during prolonged storage of the stationary phase cultures.—Old nutrient agar stocks of bacteria mutant in gene *cysH*, deficient in the reduction of 3′-phosphoadenosine 5′-phosphosulfate (PAPS) preferentially accumulate secondary mutations in genes *cysA*, *cysC*, or *cysD*. Thus, they contain secondary genetic blocks before PAPS in the cysteine biosynthetic pathway (DREYFUSS and MONTY 1963). Some old cultures are comprised predominantly of double *cys* mutants. Secondary *cys* mutations do not accumulate in mutants of the other *cys* loci studied, nor do they accumulate in frequently transferred cultures. We conclude that accumulation or continued synthesis and destruction of PAPS or some related compound in aged cultures is deleterious, leading to shortened survival in storage of singly mutant bacteria. Under these conditions, bacteria that carry a second genetic lesion effective in shutting off this flow of metabolites show preferential survival.

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