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Mutants of *Escherichia coli* with High Minimal Temperatures of Growth

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ABSTRACT

O'DONOVAN, GERARD A. (University of California, Davis), CATHERINE L. KEARNEY, AND JOHN L. INGRAHAM. Mutants of *Escherichia coli* with high minimal temperatures of growth. J. Bacteriol. **90**:611-616. 1965.—Three general classes of mutants showing increased minimal temperatures of growth have been isolated from *Escherichia coli*. These mutants do not grow at temperatures below 20 C, although their parents can grow at temperatures as low as 8 C. The first class of mutants (K-I) cannot grow below 20 C in either complex or minimal medium, but grows at nearly normal rates at 37 C on both types of media. Normal growth rate at 20 C can be conferred on these mutants by infection at a low multiplicity with a transducing phage grown on the parent. The second class of mutants (K-II) fails to grow only in minimal medium at 20 C. These mutants are characterized by their singular response to specific nutrients in minimal medium at 20 C. The third class of mutants (K-III) grows normally in minimal medium at all temperatures with either glucose or glycerol as the carbon source, but does not grow at 20 C with lactose as the carbon source.

Numerous studies have been devoted to investigation of the maximal temperature of growth of microorganisms. Mutations have been reported which result in a decrease of the maximal temperature of growth (Ingraham, 1962). In most of the cases reported, the production of a heat-labile protein accounts for the inability to grow at elevated temperatures. Edgar and Lielausis (1963), however, found mutants of phage which were unable to produce certain proteins at high temperatures.

Conversely, mutations which affect the minimal temperature of growth of microorganisms have not been extensively studied. Allen (1953) reported that stenothermophilic aerobic sporeformers frequently undergo spontaneous mutation to eurithermophily, and Azuma, Newton, and Witter (1962) reported a mutation in *Pseudomonas aeruginosa* which decreased the minimal temperature of growth. Maas (1961) described a mutant of *Escherichia coli* which synthesized ornithine transcarbamylase at 37 but not at 25 C.

In this paper, we describe the isolation and properties of mutants of *E. coli* with increased minimal temperatures of growth.

MATERIALS AND METHODS

Techniques of cultivation. Media, culture techniques, and measurements of growth rate were the same as those reported previously (Ng, Ingraham, and Marr, 1962).

Organisms. The three F^- strains of *E. coli* used (C-600-1, a prototroph; W-3018, an auxotroph requiring threonine, leucine, and vitamin B_1; and KG-531, an auxotroph requiring methionine, histidine, and vitamin B_1) were supplied by Monica Riley of this department.

Selection of mutants. Ultraviolet light (UV), ethyl methane sulfonate (EMS), 2-amino purine (AP), and *N*-methyl-*N*-nitroso-*N'*-nitroguanidine (NG) were used as mutagens. A balance of incubation time against amount of mutagen was maintained in all cases to give approximately 99.9% killing. Cells of each culture were grown overnight in glucose-limiting minimal medium at 37 C in a shaker water bath and were then transferred to a fresh medium. When the culture reached the exponential phase, mutagenic treatment was carried out as follows.

UV. Experiments with this mutagen were done according to the method of Gorini and Kaufman (1960).

EMS. An exponential culture growing in minimal medium was treated with stock EMS solution to give a 3% final concentration. Incubation in the presence of the mutagen was allowed to proceed for 2 to 2.5 hr at 37 C.

AP. An exponential culture growing in minimal medium was treated with 10 to 20 μg/ml of AP. Incubation was allowed to proceed for 3 hr at 37 C.

NG. An exponential culture growing in minimal medium was treated with 5 μg/ml of NG. Incubation was allowed to proceed for 2.5 hr at 37 C.

Phenotypic expression. After treatment with the mutagen, cultures were grown either at 37 C

in glucose-minimal medium or at 20 C in glucose-minimal medium plus appropriate supplements.

Penicillin counterselection. After phenotypic expression, cultures were inoculated into fresh glucose-minimal medium containing 20% sucrose and 0.01 M $MgSO_4$ at 20 C. After two doublings of mass, penicillin (50 to 3,000 units per ml) was added. Incubation was continued until significantly more than 50% protoplasts appeared on microscopic examination. This took about 24 hr for each of the three strains used.

The cultures were then centrifuged, suspended in glucose-minimal medium, and streaked on glucose-minimal plates at 37 C. Isolated colonies were transferred to master plates of glucose-minimal medium which were incubated at 37 C. The master plates were replicated in duplicate onto plates of glucose-minimal medium and onto plates of nutrient agar (Difco). One set of replicas was incubated at 37 C and the other at 20 C.

RESULTS

Three classes of cold-sensitive mutants developed. The first class (K-I) grows on both minimal and complex media at 37 C, but on neither at 20 C; the second (K-II) also grows on both media at 37 C, but grows only on complex medium at 20 C. The third (K-III) grows on both glucose- and lactose-minimal media at 37 C; this mutant fails to grow on lactose at 20 C, but grows normally on glucose at 20 C.

Verification of genotypes of K-I mutants. K-I mutants (17 representatives were originally isolated from 142 colonies) were compared with their parents with respect to nutritional requirements at 37 C, fermentation of lactose, susceptibility to lysis by T6 phage, and ability to mate with Hfr strains. In all respects, the mutants did not differ from their parents.

Comparison of the effect of temperature on the growth rate of K-I-01 and parent C-600-1. The specific growth rates of K-I-01, a representative of the K-I class, and its parent, C-600-1, in glucose-minimal and in complex media, are compared in the form of Arrhenius plots in Fig. 1a and 1b. In both media, the minimal temperature of growth of the mutant is slightly less than 20 C, whereas that of the wild type is considerably less (about 8 C). At 37 C, the mutant grows almost as rapidly as the wild type in both media. It is of interest that the slope in the linear portion of the Arrhenius plot is greater for the mutant than for the wild type in both media. K-I-01 is a mutant with an increased minimal temperature of growth in minimal and complex media.

Transduction experiments. Since the selection procedure used for the K-I mutants was done in liquid media, there is no evidence that these are single-step mutants. Transducibility of wild-type

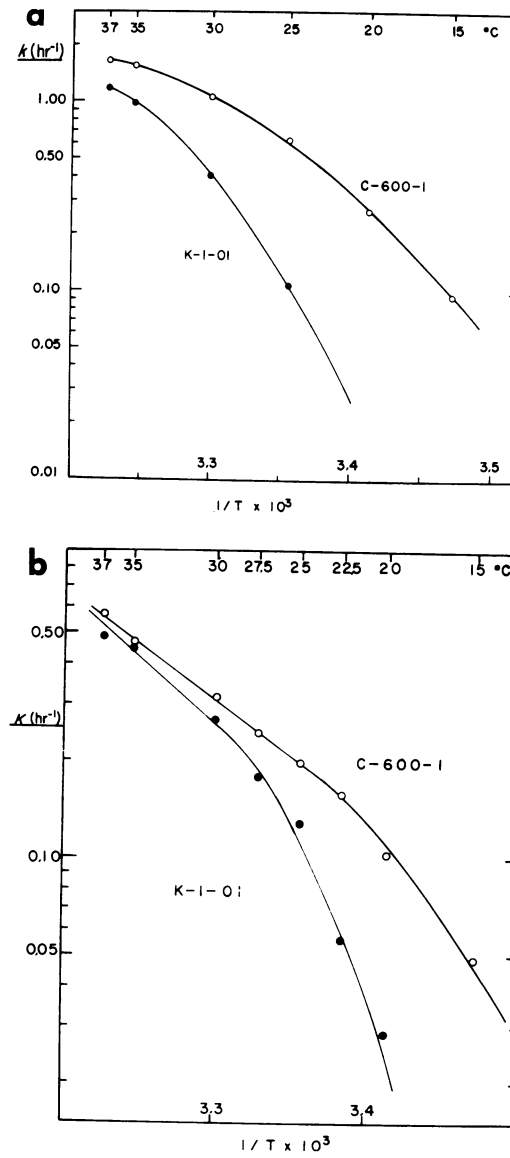

FIG. 1. *Arrhenius plot of the specific growth rate of temperature mutant K-I-01* (●) *and its parent, Escherichia coli C-600-1* (○). *The ordinate is the specific growth rate (log scale), and the abscissa is the reciprocal of the absolute temperature times 1,000.* (a) *Growth in complex medium (0.5% glucose-yeast extract—0.25% Casamino Acids).* (b) *Growth in glucose-minimal medium.*

growth response into the mutants, by low multiplicities of infection by the transducing phage, P-1, was therefore done. The results of such a transduction experiment are shown in Table 1. The frequency of transductions is 1.1×10^{-4}.

TABLE 1. *Transduction of wild-type growth into K-I-01**

Hr held at 30 C	Colonies developing after 7 days at 20 C	
	Control	P-1 infected
0	0, 0	36, 33
1	0, 4	32, —
2	0, 0	24, 30
4	0, 7	25, 44

* K-I-01 was grown in L-broth to a density of 250 μg/ml (dry weight), diluted 1:1 in L-broth with 2×10^{-3} M $CaCl_2$ and 0.1 M tris(hydroxymethyl)-aminomethane-acetate buffer at pH 7, distributed in 1-ml amounts into two test tubes, and allowed to stand at room temperature for 30 min. During this time, cell numbers in the dilution were estimated by plating appropriate dilutions on nutrient agar plates which were incubated at 37 C. The sterility of the P-1 stock was confirmed by plating a 10^{-3} dilution on nutrient agar plates which were incubated at 20 C. After the 30-min period, 0.05 ml of P-1 stock (4×10^{10} plaque-forming units per milliliter), which was grown on C-600-1, was added to one tube (P-1 infected), and the other served as a control. After 15 min at 37 C, the contents of both tubes were plated at a 10^{-3} dilution onto a series of plates. All plates were held at room temperature for 2 hr and then transferred to 30 C. At the indicated times, two control and two experimental plates were transferred to 20 C and were incubated for 7 days. Initial number of bacteria was 3.0×10^9 per milliliter.

K-II-type mutants. K-II mutants grow on complex medium at 20 and 37 C, but grow only at 37 C on minimal medium. However, the addition of small molecules to the minimal medium at 20 C allows the growth of all K-II mutants. Several different mutants have been isolated which require a wide variety of small molecules at low temperature. The majority of mutants are auxotrophic for a single nutrient at low temperature; however, certain mutants are auxotrophic for two or more nutrients. The two strains of *E. coli* used, C-600-1 and W-3018, both yielded mutants (Table 2). These data indicate that C-600-1, a prototroph, according to our methods of selection, yielded mutants in greater frequency than W-3018, which is auxotrophic for threonine, leucine, and vitamin B_1. EMS gave more mutants than the other mutagens tested. AP yielded the most stable mutants, as judged by rate of reversion.

Greater numbers of K-II mutants were obtained if phenotypic expression was carried out at 37 rather than 20 C (Table 3). No K-II mutants were isolated without counterselection with penicillin.

Since different amounts of penicillin have been employed as a counter-selecting agent, both here and elsewhere, a general study was carried out to see if an optimal concentration of penicillin is possible. Several concentrations from 50 to 3,000 units per ml were tried. Best results were obtained by use of at least 2,000 units per ml. However, a more important index was the formation of protoplasts, as observed microscopically. No mutants were isolated if samples were taken before protoplasts appeared. In more recent studies, without the use of sucrose and $MgSO_4$, the observation of lysis was the criterion used.

Two major problems arise when K-II mutants are being sought. The first is the plurality of K-I

TABLE 2. *K-II type mutants of Escherichia coli*

Mutagen	Strain	Total colonies	Total mutants	Per cent
EMS*	C-600-1	664	65	9.8
AP†	C-600-1	332	12	3.6
NG‡	C-600-1	249	7	3.1
EMS	W-3018	332	3	1.0
AP	W-3018	166	4	2.4
NG	W-3018	83	0	0

* Ethyl methane sulfonate.
† 2-Amino purine.
‡ *N*-methyl-*N*-nitroso-*N'*-nitroguanidine.

TABLE 3. *Effect of mutagen and phenotypic expression on the isolation of K-II mutants of Escherichia coli*

Temp of phenotypic expression	Mutagen	Strain	Total colonies	Total mutants	Per cent
C					
37	EMS*	C-600-1	498	56	11.4
	AP†	C-600-1	249	8	3.2
	NG‡	C-600-1	166	7	4.2
	EMS	W-3018	166	3	1.8
	AP	W-3018	83	4	4.9
	NG	W-3018	83	0	0
20	EMS	C-600-1	166	9	5.4
	AP	C-600-1	83	4	4.8
	NG	C-600-1	83	0	0
	EMS	W-3018	166	0	0
	AP	W-3018	83	0	0
	NG	W-3018	—	—	—

* Ethyl methane sulfonate.
† 2-Amino purine.
‡ *N*-methyl-*N*-nitroso-*N'*-nitroguanidine.

mutants which appear and which must be avoided. This is done by picking only those colonies which grow on complex medium at 20 C. The second problem arises from the frequent appearance of auxotrophic mutants which can be avoided by ensuring that those colonies which grow on complex medium at 20 C also grow on minimal medium at 37 C.

Three general classes of K-II mutants have been isolated. (i) Mutants which require vitamin(s) or cofactor(s) for growth at low temperature have been obtained. Four mutants of this class have been isolated from *E. coli* W-3018 by the AP treatment. Three of the mutants require more than one vitamin for growth at low temperature, and the fourth specifically requires pyridoxine. (ii) A second general class of mutants has been isolated which requires more than one small molecule for growth. On further analyses, these were found to be auxotrophic mutants, requiring, in addition, another small molecule at low temperature. An example of this class is a mutant which is auxotrophic for proline and which further requires phenylalanine at 20 C. However, the growth rate of this mutant on minimal medium supplemented with proline at 37 C was not the normal for wild-type rate. The differential growth rates at 20 and 37 C were, therefore, not sufficient to allow the analyses of the enzymatic deficiencies of this mutant. (iii) The third and most valuable class of mutants isolated was that class of mutants which required only one supplement—that of a small molecule—at low temperature. At this point, only amino acid-requiring mutants were sought, and 107 such mutants were isolated. On further characterization, 75% of these mutants were found to require histidine at 20 C. This was an unexpected result; 17 of these have now been purified and rechecked, and all are extremely stable histidine-requiring mutants at 20 C. All 17 grow on high concentrations (100 mM) of histidinol at 20 C. Fifteen of the remainder required arginine at 20 C, 9 required methionine, 2 required lysine, 2 required isoleucine, and 1 required leucine. Owing to their inability to grow at wild-type rate in glucose-minimal medium at 37 C, the latter group of 15 were not further studied.

A typical mutant of the K-II class is K-II-27. The growth rates of this mutant and of its wild-type parent, C-600-1, are compared in the form of Arrhenius plots in Fig. 2. In complex medium, the mutant and the wild type grow at the same rate at all temperatures (Fig. 2a). In glucose minimal medium, the ratio of the growth rate of wild type to mutant increases progressively as the

TABLE 4. *Growth rates (k) of K-III type mutants and their parent Escherichia coli KG-531*

Strain	Growth at				
	37 C on		27 C on	20 C on	
	Glucose	Lactose	Lactose	Glucose	Lactose
Parent KG-531	0.774	0.520	0.374	0.170	0.127
Mutant K-III-01	—	—	0.062	0.168	0.036
Mutant K-III-04	0.748	0.521	0.026	0.159	0.000

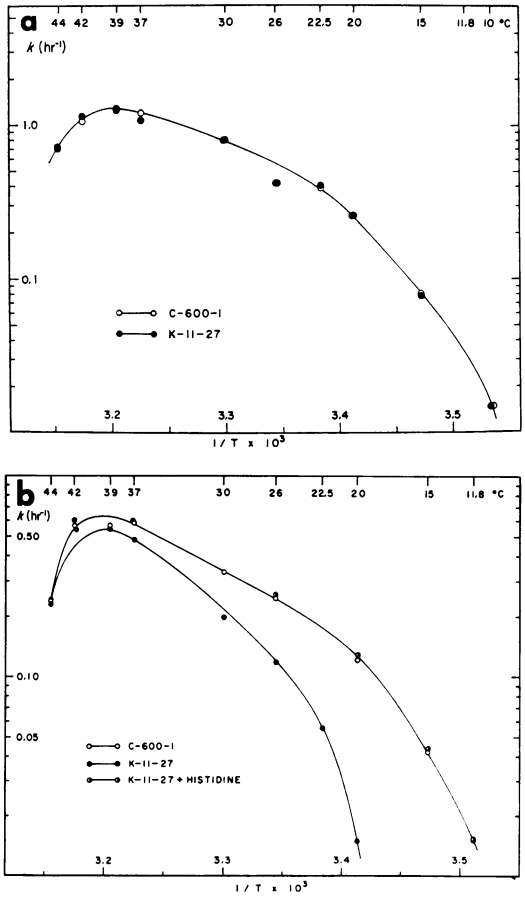

FIG. 2. *Arrhenius plot of the specific growth rate of temperature mutant K-II-27* (●) *and its parent, Escherichia coli C-600-1* (○). *The ordinate is the specific growth rate (log scale), and the abscissa is the reciprocal of the absolute temperature times 1,000.* (a) *Growth in nutrient broth.* (b) *Growth of K-II-27 in glucose-minimal medium plus 10 μg/ml of histidine* (◑).

temperature of growth is decreased. If glucose minimal medium is supplemented with histidine, K-II-27 grows at the same rate as the wild type at all temperatures (Fig. 2b). K-II-27 is a mutant which has a specific requirement for histidine only at low temperature.

K-III type mutants. K-III mutants were isolated from the parental strain KG-531 by the AP treatment described in Materials and Methods. A typical mutant K-III-01 grows normally with glucose as the carbon source at both 20 and 37 C in minimal medium, but it fails to grow at 20 C when lactose is the carbon source. Both β-galactosidase and galactoside permease are inducible at 20 C by high levels of isopropyl-thio-β-D-galactopyranoside and methyl-thio-β-D-galactopyranoside. Enzyme and permease produced at either high or low temperature by the mutant are not inactivated at low temperature.

Growth rates of K-III mutants compared with their wild-type parent are shown in Table 4.

DISCUSSION

Broad general comparisons of mesophiles and psychrophiles have not established a basis for difference in minimal temperature of growth. This difference is only one of the many which exist between these two distinct classes of microorganisms; consequently, it is difficult to assign a causal role to any experimentally observed difference. The mutants described here obviate such mesophile-psychrophile comparisons, because the difference in minimal temperature of growth between parents and mutants appears to result from a single genetic change. It is significant that K-I, K-II, and K-III mutants all differ phenotypically from their parents by (i) ceasing to grow at a higher minimal temperature, and by (ii) exhibiting an increased temperature characteristic in the linear portion of the Arrhenius plot of growth rate. The same differences exist between mesophiles and psychrophiles (Ingraham, 1958).

Mutations which decrease the maximal temperature of growth (temperature-sensitive mutants) result from lesions which either decrease the stability of certain proteins at high temperature (Maas and Davis, 1952; Horowitz and Fling, 1953) or make them particularly sensitive to elevated temperatures during the period of synthesis (Edgar and Lielausis, 1964). There are also reports of mutations in which repressors have apparently become labile at elevated temperatures (Novick, Lennox, and Jacob, 1963; Gallant and Stapleton, 1963). However, it is improbable that the mutants described here (cold-sensitive mutants) result from changes which render proteins unstable at low temperatures. (If such were the explanation, the proteins would have to be appreciably cold-labile at 30 C, since at this temperature the growth rates of the mutants are less than those of their parents.) In fact, in all cases studied (Ingraham and Kearney, *unpublished data*; O'Donovan and Ingraham, *unpublished data*), cold-sensitive mutants produce proteins which are not unstable even at 20 C.

Temperature-sensitive mutants, by providing biochemical blocks, promise great utility for elucidating the pathways and, indirectly, the regulation of macromolecule biosynthesis (Eidlic and Neidhardt, 1965) in that they allow one to study lesions which would normally be lethal. We feel that cold-sensitive mutants will provide particularly useful tools for the study of the regulation of biosynthesis (in addition to their obvious use as tools for the study of minimal temperature of growth), since the lesions involved probably affect regulatory mechanisms directly.

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