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Genetic Mapping of Cold Resistance Gene of *Escherichia coli*

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Using cold resistant mutants, MET1 and MET2, obtained from *Escherichia coli* K-12, genetic mapping of the cold resistance gene(s) of *E. coli* was performed by the conjugation and transduction techniques. The gene(s) was confirmed to be located close to *trpB* at 28 min (revised chromosome linkage map, 1983) on the *E. coli* chromosome.

In a previous paper,[1] we described the isolation and some properties of the cold resistant mutants, MET1 and MET2, from *Escherichia coli* K-12 (ATCC 10798). To elucidate the physiological and biochemical functions of the gene(s) concerned in the cold resistance of *E. coli*, genetic analysis of these mutants is very significant.

In this paper, we report the genetic mapping of the cold resistance gene(s) of *E. coli*.

MATERIALS AND METHODS

Strains and phages used. The *E. coli* K-12 strains used in this study are listed in Table I. The gene symbol for cold resistance is *crg* (cold resistant growth) according to the recommendation of Demerec *et al.*[3] The genotypes or phenotypes of the parent (K-12) and mutant (MET1, MET2) strains are represented as crg^+ and crg^- or Csg and Crg, respectively. The methods used for strain construction or manipulation of genetic markers are also indicated in Table I or in the text. Figure 1 shows the genetic map locations of relevant genetic markers and Hfr points of origin.

The phages, P1*kc* (IFO 20008), MS2 (IFO 20015) and λ_{vir} (IFO 20017), were obtained from the Institute for Fermentation, Osaka, Japan.

Media and chemicals. Nutrient broth contained 10 g of polypepton (Daigo Eiyo Kagaku, Co., Osaka), 3 g of meat extract (Wako Pure Chemical Industries, Osaka) and 5 g of NaCl per liter (pH 7.2). EMB (eosin-methylene blue) agar to test for lactose utilization was obtained from Eiken Chemical Co., Tokyo. These complex media were routinely supplemented with thymine (40 μg/ml) and thiamine (10 μg/ml). L broth contained 10 g of polypepton, 5 g of yeast extract (Oriental Yeast Co., Osaka), 5 g of NaCl and 1 g of glucose per liter (pH 7.2). The minimal medium used was that of Davis and Mingioli,[4] containing 0.2% (w/v) glucose as a carbon source. When necessary, Vitamin-free Casamino Acids (0.2%; Difco Laboratories Ltd., Detroit, Mich.), L-amino acids (40 μg/ml), thymine (40 μg/ml), thiamine (10 μg/ml) or streptomycin (100 μg/ml) was added to the minimal medium. The media containing 1.5% agar (Difco) were used for plating experiments.

Streptomycin sulfate and lysozyme were obtained from Meiji Seika Co., Tokyo, and Sigma Chemical Co., St. Louis, Mo., respectively.

Growth conditions. A stationary-phase culture was obtained by static incubation in 10 ml of L broth at 37°C overnight. The viable cell counts of the cultures were about 2×10^9 cells per ml. To obtain a log-phase culture, 0.2 ml of the stationary-phase culture was inoculated into 10 ml of fresh L broth in a test tube (18 mmϕ × 200 mm) and incubated at 37°C with reciprocal shaking (124 spm; 3.6 cm), unless otherwise stated, to a density of 2 to 3×10^8 cells per ml.

Determination of $CFU_{7.5}$, $fCFU_{7.5}$ and survial at 7.5°C. Stationary-phase cultures of the strains tested were diluted with saline and 200 to 300 viable cells (as determined by incubating at 30°C overnight) were spread on a nutrient agar medium plate, unless otherwise stated. The plates were incubated for 20 to 40 days at 7.5°C (±0.2°C) and the average number of colonies from five to ten plates was determined as $CFU_{7.5}$ (colony-forming units at 7.5°C) for each strain. These plates were then in-

TABLE I. LIST OF STRAINS USED

Strain	Genotype[a]	Derivation[b]	Source
K-12	F^+ λ^+ λ^r wild type		ATCC 10798
MET1	Same as K-12, except *crg-1*	DAPA mutagenesis of K-12	S. Kawamoto[1)]
MET2	Same as K-12, except *crg-2*	UV mutagenesis of K-12	S. Kawamoto[1)]
MET3	Same as K-12, except *lacY1 trpB* λ^-	Pro^+ Trp^- His^+ Thy^+ recombinant from ST5 (donor) × *pro* strain of K-12	This study
JE1031	HfrH *metB thi* λ^-		S. Mizushima
SK101	Same as JE1031, except *crg-1*	P1 (MET1)→JE1031, Crg selection	This study
SK102	Same as JE1031, except *crg-2*	P1 (MET2)→JE1031, Crg selection	This study
P4X8	HfrP4X *metB thi* λ^-		S. Mizushima
SK81	Same as P4X8, except *crg-1*	P1 (MET1)→P4X8, Crg selection	This study
SK82	Same as P4X8, except *crg-2*	P1 (MET2)→P4X8, Crg selection	This study
PA3092	F^- *thr-1 leu-6 tonA2 lacY1 supE44 trpB his-1 thyA str-9 malA1 xyl-7 mtl-2 argH1 thi-1* λ^-		S. Mizushima
ST5	Same as PA3092, except *argH*$^+$ *thr*$^+$ *leu*$^+$ HfrH	Arg^+ $MS2^s$ Str^r recombinant from JE1031 (donor) × PA3092	This study

[a] Symbols for genetic markers, see Bachmann.[2)] *crg* is the symbol for cold resistant growth.
[b] DAPA, sodium *p*-dimethylaminophenyldiazosulfonate. UV, ultraviolet rays.

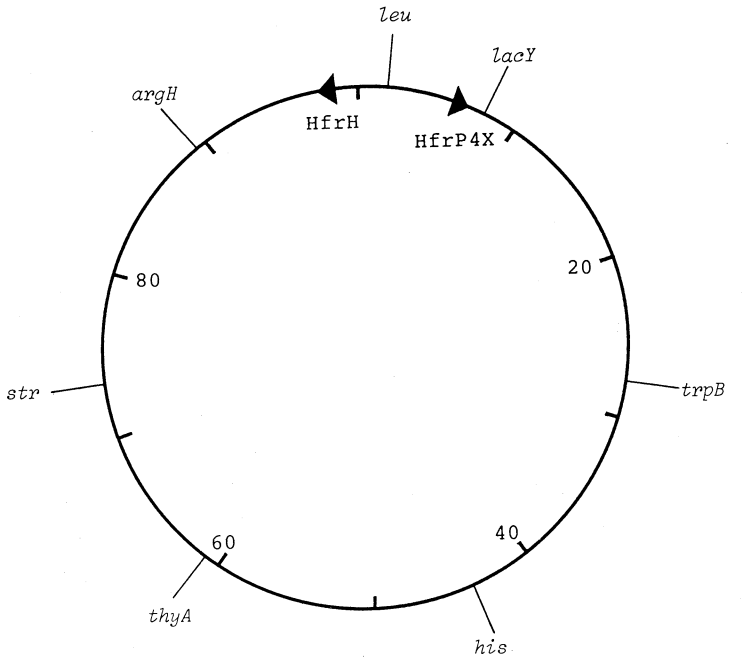

FIG. 1. Genetic Map of *E. coli* K-12 Showing the Locations of Some Relevant Markers, and the Origins and the Directions of Transfer of the Chromosome of Hfr Strains.

cubated for 2 days at 30°C, and survival (%) at 7.5°C was calculated. The fraction of $CFU_{7.5}$ ($fCFU_{7.5}$) at 7.5°C is given as a percentage of the average number of initial viable cells. There was essentially no difference between survival and $fCFU_{7.5}$ after 25 days at 7.5°C as determined in the above manner for the cold resistant mutants, MET1 and MET2.

Conjugation. Donor strains were grown to the log-phase without shaking.[5)] The log-phase cultures of donor (0.5 ml) and recipient (5 ml) strains were mixed with 5 ml of fresh L broth prewarmed at 37°C in a 300 ml

Erlenmeyer flask and incubated at 37°C without shaking for the desired time of mating. The donor to recipient cell ratio was usually between 1 : 10 and 1 : 20. The total bacterial density was no more than 2×10^8 cells per ml at the commencement of mating.[6] The cells of the mating mixture were then vortexed vigorously for 30 sec, sedimented and suspended in saline for plating.

Preparation of phage P1kc lysate. The log-phase culture of a donor strain in L broth containing 2.5 mM $CaCl_2$ at 37°C was infected with phage P1*kc* at a multiplicity of about 2, and the shaking continued until lysis was complete (about 2 to 3 hr after addition of the phage). Lysozyme (200 μg/ml) and chloroform (about 1/500 volume) were added, and the shaking continued for another 30 min. Cell debris was then removed by centrifugation at $15{,}000 \times g$ for 20 min at 4°C. The cleared lysate was passed through a membrane filter (25 mm diameter, 0.8 μm pore size; Toyo Roshi Co., Tokyo) and stored at 4°C. Titers ranging from 3 to 6×10^9 plaque-forming units per ml were routinely obtained. Phage P1*kc* was recycled on the same donor at least twice before being used for transduction.

Transduction. A stationary-phase culture sample (5 ml) of a recipient strain was centrifuged, and the cells were resuspended in the same volume of fresh L broth containing 2.5 mM $CaCl_2$. Phage P1*kc* was added at a multiplicity of about 0.2. After 25 min at 37°C, the cells were sedimented, washed once with 1% sodium citrate solution in order to prevent readsorption of phage and then suspended in saline for plating.

Selection of Crg recombinants and Crg transductants. Crg transductants and Crg recombinants from strain PA3092 were selected by plating on minimal agar medium supplemented with Casamino Acids, tryptophan, thymine, thiamine and streptomycin, and incubating for 60 days at 7.5°C. Crg transductants from strains JE1031 and P4X8 were selected in the same manner, except that the selective medium used was nutrient agar. Spontaneous mutation frequencies for Crg of the parent strains were usually less than 5×10^{-8}.

Unselected markers. Recombinants and transductants were purified once on the original selective medium and stored on a master plate of the same medium (usually 50 clones per plate).

To test for the presence of a cold resistance gene (*crg*$^-$) or nonresistance gene (*crg*$^+$) as an unselected marker, a master plate was replicated onto three plates of nutrient agar medium followed by incubation for 15 or 25 days at 7.5°C when Trp$^+$ transductants from strain MET3 or PA3092 were tested, respectively. By this method, it was possible to distinguish cold resistant clones (Crg) from nonresistant clones (Csg). That is, Crg clones showed heavy growth in patches on all the replicated plates, whereas Csg clones showed little growth on the plates.

Strain construction. Strain MET3 was constructed from strain K-12 by the following series of steps. Strain ST5 was constructed from strain PA3092 by mating (2.5 hr) with Hfr strain JE1031 (selecting Arg$^+$, streptomycin resistance and male specific phage MS2 sensitivity). A proline auxotroph mutant was isolated from strain K-12 by penicillin treatment as described by Miller.[7] This strain was then mated with Hfr strain ST5 for 1 hr, and a *pro*$^+$ *his*$^+$ *thyA*$^+$ recombinant was isolated that retained the *lacY* and *trpB* markers of strain ST5. This isolate was called MET3. Strain MET3 was resistant to phage λ_{vir} (λ^r) similar to its parent, but it did not show induction of λ prophage by ultraviolet rays[8] and was regarded as λ^-.

RESULTS AND DISCUSSION

Cold resistance of mutants MET1 and MET2

By ultraviolet ray and sodium *p*-dimethylaminophenyldiazosulfonate mutageneses, the mutants, MET1 and MET2, with the ability to grow at 7.5°C (±0.2°C) on nutrient agar medium ($fCFU_{7.5} = 90 \sim 100\%$ after 25 days) were obtained from strain K-12 showing rapid loss of viability under the same conditions. The cold resistance was shown to be stably inherited in these mutants in a previous study.[1]

It was observed that strains MET1 and MET2 were able to grow at 7.5°C on L agar ($fCFU_{7.5} = 90 \sim 100\%$ after 20 days) and minimal agar supplemented with Casamino Acids ($fCFU_{7.5} = 90 \sim 100\%$ after 30 days) media, but not on minimal agar containing glucose as a sole carbon source and Bacto-Penassay (Difco) agar media ($fCFU_{7.5} = 0\%$ after 40 days). The mutants, however, showed about 7-fold and 40-fold higher survival than the parent, K-12, after 20 days at 7.5°C on minimal agar (MET1 and MET2, about 40%; K-12, 6%) and Bacto-Penassay agar (MET1 and MET2, about 20%; K-12, 0.5%), media, respectively. The reason why the mutants could not grow on a complex medium such as Bacto-Penassay agar is unclear. It was recognized that the mutants could not grow and showed loss of viability on all the above media at 6°C (±0.2°C).

Conjugational mapping of crg-1 and crg-2

The ability to grow at 7.5°C on nutrient agar medium or minimal agar medium supplemented with Casamino Acids was used as a phenotype for the cold resistance mutations, *crg-1* and *crg-2*. To detect the cold resistance in recombinants, it was necessary to use Hfr donors carrying *crg-1* or *crg-2*. In order to introduce *crg-1* or *crg-2* of mutants MET1 and MET2 into Hfr strains JE1031 and P4X8, the four transduction crosses, *i.e.* i) P1 (MET1) × JE1031, ii) P1 (MET2) × JE1031, iii) P1 (MET1) × P4X8 and iv) P1 (MET2) × P4X8, were first performed. Crg transductants were selected on nutrient agar medium after incubation for 60 days at 7.5°C. Transduction frequencies (transductants per input phage) for Crg were 10^{-6} to 10^{-5} in the crosses. One of the Crg transductants obtained in each cross was isolated and purified twice on the same medium at 30°C. The resulting four clones were all Met$^-$, Thi$^-$ and sensitive to male specific phage MS2, similar to the parents. These clones were all able to grow at 7.5°C on nutrient agar medium ($fCFU_{7.5} = 90 \sim 100\%$ after 30 days), whereas the parents showed rapid loss of viability under the same conditions. The results indicate that the *crg-1* or *crg-2* mutation is stably inherited in the transductants.

These transductants, SK101 (HfrH *crg-1*), SK102 (HfrH *crg-2*), SK81 (HfrP4X *crg-1*) and SK82 (HfrP4X *crg-2*), were then allowed to mate for 2 hr with strain PA3092 carrying multiple auxotrophic markers (see Table I). Crg (Str^r) recombinants were selected on minimal agar medium supplemented with Casamino Acids, tryptophan, thymine, thiamine and streptomycin after 60 days at 7.5°C, and scored for unselected markers. The results are presented in Table II. Recombination frequencies (recombinatants per input Hfr cell) for Crg were about 10^{-4} and 10^{-5} in crosses 1 and 2, and crosses 3 and 4, respectively. When HfrH strains, SK101 (*crg-1*) and SK102 (*crg-2*), were the donors, the *crg* mutations were coinherited with *trpB* and *his-1* at relatively high frequencies. Linkage values of 95 and 75% or 92 and 66% were obtained for *crg-1* or *crg-2*, respectively. When HfrP4X strains, SK-81 (*crg-1*) and SK82 (*crg-2*), were the donors in which the *trpB* gene was distal to the origin of chromosomal transfer (see Fig. 1), the *crg* mutations were also coinherited with *trpB* at relatively high frequencies, and the same linkage values (88%) were obtained for *crg-1* and *crg-2*. These results suggest that *crg-1* and *crg-2* are both located near *trpB*.

Linkage of crg-1 and crg-2 to the trpB marker

The linkage of *crg-1* and *crg-2* to the *trpB* marker was tested by P1*kc* transduction. P1*kc* lysates were prepared from the mutants, MET1 (*crg-1 trpB*$^+$) and MET2 (*crg-2 trpB*$^+$). First, each lysate was applied to strain PA3092 (*crg*$^+$ *trpB*), and Crg transductants were selected at 7.5°C in the same manner as described above and scored for tryptophan requirement.

TABLE II. UNSELECTED MARKER ANALYSIS OF Crg RECOMBINANTS

Cross No.	Conjugation[a] Donor × Recipient	Selected marker	No. of recombinants tested	Frequency of unselected marker (%)					
				leu$^+$	*lacY*$^+$	*trpB*$^+$	*his*$^+$	*thyA*$^+$	*argH*$^+$
1	SK101 (HfrH) × PA3092	*crg-1* (*str-9*)[b]	150	14	33	95	75	5	2
2	SK102 (HfrH) × PA3092	*crg-2* (*str-9*)	282	7	20	93	66	4	2
3	SK81 (HfrP4X) × PA3092	*crg-1* (*str-9*)	232	5	3	88	30	13	2
4	SK82 (HfrP4X) × PA3092	*crg-2* (*str-9*)	153	3	3	88	27	12	0

[a] The time of mating was 2 hr. Crg recombinants were selected by plating on minimal agar medium supplemented with Casamino Acids, tryptophan, thymine, thiamine and streptomycin, and incubating for 60 days at 7.5°C, and scored for the unselected markers. All Hfr strains are *leu*$^+$ *lacY*$^+$ *trpB*$^+$ *his*$^+$ *thyA*$^+$ *argH*$^+$ *crg*$^-$ (*crg-1* or *crg-2*) and streptomycin sensitive; PA3092 carries *leu-6 lacY1 trpB his-1 thyA argH1 crg*$^+$ *str-9*.

[b] The marker in parentheses is derived from the recipient and counterselective against the donor.

TABLE III. LINKAGE BETWEEN *crg* MUTATIONS AND *trpB* MARKER

Cross No.	P1 Donor	Recipient	Selected marker	Unselected[a] marker	Cotransduction frequency (%)	No. unselected/ No. selected
1	MET1 (*crg-1 trpB*$^+$)	PA3092 (*crg*$^+$ *trpB*)	*crg-1*	*trpB*$^+$	51	263/511
			trpB$^+$	*crg-1*	37	87/234
2	MET2 (*crg-2 trpB*$^+$)	PA3092 (*crg*$^+$ *trpB*)	*crg-2*	*trpB*$^+$	46	113/248
			trpB$^+$	*crg-2*	38	90/238
3	MET1 (*crg-1 trpB*$^+$)	MET3 (*crg*$^+$ *trpB*)	*trpB*$^+$	*crg-1*	48	120/250
4	MET2 (*crg-2 trpB*$^+$)	MET3 (*crg*$^+$ *trpB*)	*trpB*$^+$	*crg-2*	50	100/200

[a] The *crg*$^-$ of Trp$^+$ transductants as an unselected marker was scored by replicating on nutrient agar medium and incubating for 15 or 25 days at 7.5°C when strain MET3 or PA3092 was the recipient, respectively.

The transduction frequency for Crg was about 10^{-6} in each cross. The results in Table III (crosses 1 and 2) show that *crg-1* and *crg-2* were both cotransducible with *trpB* at frequencies of 51% and 46%, respectively. Second, each lysate was applied to strain PA3092 and strain MET3 which was a *trpB* derivative of strain K-12 (see details of the strain construction in MATERIALS AND METHODS). Trp$^+$ transductants were selected at 37°C and tested for cold resistance by the replica plating method as described in MATERIALS AND METHODS. The transduction frequency for Trp$^+$ was about 5×10^{-5} in each cross. The results in Table III (crosses 1 to 4) show that the cotransduction frequencies of *trpB* and *crg-1*, and *trpB* and *crg-2* were 37 and 38% when strain PA3092 was the recipient or 48 and 50% when strain MET3 was the recipient, respectively. From the data shown in Table III, it was concluded that the cold resistance mutations are both located close to *trpB* at 28 min on the *E. coli* genetic map of Bachmann.[2)]

In order to elucidate the physiological and biochemical functions of the *crg* gene(s), extensive physiological studies at low temperatures are in progress using *crg* transductants and the parent which are isogenic, apart from the *crg* gene(s).

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