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The characterization of an alanine racemase mutant of *Escherichia coli*

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SUMMARY

Among temperature-sensitive mutants of *Escherichia coli* a strain was discovered requiring D-alanine for growth. It was proved to possess an altered alanine racemase. The structural gene for this enzyme, designated *alr*, is located between *metB* and *purA*. The properties of the enzyme and its locus suggest that it is not under control of the mechanisms which regulate mucopeptide formation. A suppressor of the *alr* mutation was discovered near *trp*, and termed *msuA*.

1. INTRODUCTION

Among heat-sensitive mutants of *Escherichia coli* K-12, several showed lysis at the restrictive temperature (A. Rörsch, unpublished). It was expected that in these strains steps in cell-wall synthesis were blocked, and they were accordingly studied in detail. Since D-alanine is a component of the mucopeptide layer of the cell wall, one mutant in which a low concentration of D-alanine prevented lysis was biochemically and genetically investigated. Because D-alanine is synthesized from L-alanine by the enzyme D-alanine:L-alanine racemase, the temperature-sensitive mutant was expected to have its alanine racemase activity impaired at 42 °C.

2. MATERIAL AND METHODS

(i) *Bacterial and phage strains*

The strains used are listed in Table 1. Mutants showing lysis are denoted by the symbol TKL. The TKL strains were induced by mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine of KMBL158, a descendant of CR34 (Okada, Yanagisawa & Ryan, 1960). A low concentration of D-alanine in the medium restored growth in TKL10, TKL25 and TKL51. The mutations in the latter two mutants being leaky, TKL10 was chosen for further study.

(ii) *Media*

The media employed have been described (Wijsman, 1972). For the selection of thermoresistant recombinants a medium was used containing, per litre: 1·5 g Na_2HPO_4, 0·75 g KH_2PO_4, 0·5 g NH_4Cl, 0·05 g $MgSO_4$. Streptomycin was added to

Table 1. *Strains of* Escherichia coli *K-12*

Strain	Sex	Genetic characters
KMBL146	F⁻	*thr leu his ilvA arg thi pyrF thyA lac tonA tsx str*
KMBL158	F⁻	KMBL146 *trp*
TKL10	F⁻	KMBL158 *alr*
GIA22	F⁻	KMBL146 *thy*⁺
X478	F⁻	*lys trp met pro leu purE thi lac ara tsx tonA str*
H731	F⁻	*his tyrA trp thi purB pyr lac gal xyl tonA tsx str*
H787	Hfr R4	*pyrA purA metB*
B9	Hfr B9	*metB*
KMBL171	HfrH	*met*
PC0433	Hfr P4X6	*met thi*
GIA26	Hfr P4X6	*purE xyl str*

Genetic symbols are those of Taylor (1970). GIA26 is a recombinant from the conjugation PC0433 × X478.

a concentration of 100 μg/ml. As a source of D-alanyl-D-alanine, the racemic mixture D,L-alanyl-D,L-alanine (Sigma) was used.

(iii) *Conjugation and transduction methods*

The methods used in genetic experiments have been described previously (Wijsman, 1972).

(iv) *Assay of alanine racemase*

D-alanine:L-alanine racemase activity was assayed essentially as described by Berberich, Kaback & Freese (1968). The cells were washed twice in 0·05 M Tris*-HCl (pH 8·0), concentrated × 100 and ultrasonically disrupted. The lysate was centrifuged at 30000 *g* for 20 min at low temperature; no activity was present in the pellet. The crude supernatant was assayed for its ability to form D-alanine from 15 mM L-alanine, during 15 min in a volume of 3 ml 0·05 M Tris-HCl (pH 8·0) containing 0·1 mM pyridoxal phosphate. After 10 min boiling and centrifuging, the D-alanine formed is then converted to pyruvate by 1 mg D-amino acid oxidase (Boehringer) in a volume of 3·3 ml Tris-HCl during 90 min with vigorous aeration. After further boiling for 10 min and centrifuging, the pyruvate is finally determined by adding lactate dehydrogenase (2·5 μg per 3·3 ml, in Tris-HCl containing 0·1 mM NADH) and measuring the decrease in extinction at 340 nm.

Activity is expressed as μmoles D-alanine per mg protein formed in 15 min. Protein was determined spectrophotometrically as described by Waddell (1956). For induction, the cells were grown for 2 h on 0·5 % alanine (either the L or the D stereoisomer) as sole carbon source.

* Abbrevations: Tris = trishydroxymethylaminomethane; *alr* = gene for alanine racemase; *msuA* = missense suppressor A; this nomenclature was suggested by Dr A. L. Taylor.

Table 2. *Inducibility of alanine racemase activity in strain TKL 10 and other related strains*

Strain	Temperature 28 °C	37 °C	42 °C
(*a*) No induction			
KMBL146	0·081	—	0·085
GIA22	0·106	0·099	0·099
TKL10	0·009	—	0·002
(*b*) Induction by L-alanine			
GIA22	0·654	0·743	0·777
TKL10	0·012	—	0·001
(*c*) Induction by D-alanine			
GIA22	—	0·740	—
TKL10	0·008	—	0·009

Induction was assessed by assay of racemase activity after 2 h growth with 0·5 % alanine as sole carbon source.

3. RESULTS

(i) *Growth tests*

The growth of TKL10 at 28 °C was normal, but at 42 °C rapid lysis occurred. However, when 10 % sucrose was added, no lysis occurred, and electron micrographs show that spheroplasts were formed (Wijsman, 1970). The addition to solid medium of either 2 % NaCl, 20 % sucrose or 40 μg/ml D-alanine restored the colony-forming ability at 42 °C; D-alanyl-D-alanine had the same effect.

(ii) *Enzymic studies*

Racemase activity was determined as described under Material and Methods. It is remarkable that the use of alanine as sole carbon source resulted in induction of the enzyme in the wild type (Table 2). At 28 °C and at 42 °C the basal as well as the induced activity in TKL10 was markedly decreased compared with the parent strain KMBL146 and the related strain GIA22. The D-alanine deaminating activity of TKL10, used as a reference, was normal (unpublished data).

Rosso, Takashima & Adams (1969) discuss grounds for assuming that in *Pseudomonas putida* the *in vivo* function of the racemase is the catabolism of L-alanine via D-alanine, including the significant observation that when the racemase is blocked (by a chemical inhibitor) growth on D-alanine is still possible, while growth on L-alanine is absent. In the present study the same phenomenon was also demonstrated for TKL10 at 42 °C. Although D-alanine (0·5 %) served as a carbon source at 28 °C as well as at 42 °C, at neither temperature could L-alanine be used as a carbon source, in contrast to the situation in KMBL158. This appears to be due to a second mutation in TKL10, since the ability to utilize L-alanine at 28 °C could be restored by transduction independently of the thermosensitive D-alanine auxotrophy. After the ability to use L-alanine as sole carbon source at 28 °C has been recovered, the presence of the *alr* mutation still prevents growth on

Table 3. *Analysis of recombinants after mating TKL 10 with different Hfr strains*

Cross	F⁻	Hfr	Selected marker	No. of recombinants tested	Unselected marker(s)	No.	Linkage (%)
1	TKL 10 *alr⁻ msuA⁺ pyrF⁻*	KMBL 171 (HfrH) *alr⁺ msuA⁻ pyrF⁺*	*pyrF⁺*	40	Alr⁺(*msuA⁻*)	37	92
2	TKL 10 *alr⁻ msuA⁺ pyrF⁻ trp⁻*	B 9 (HfrB9) *alr⁺ msuA⁻ pyrF⁺ trp⁺*	Alr⁺ (*msuA⁻*)	79	*trp⁺ pyrF⁺*	66	—
					trp⁺ pyrF⁻	5	—
					trp⁻ pyrF⁻	8	—
					trp⁻ pyrF⁺	0	—
			Alr⁺ (*msuA⁻*)	79	*pyrF⁺*	66	83
					trp⁺	71	92

Two Hfr strains were mated with TKL 10 in nutrient broth for 3 h and *PyrF⁺* or Alr⁺ recombinant colonies selected on minimal medium at 42 °C. Alr⁺ colonies may have inherited the *alr⁺* or *msuA⁻* donor allele, the latter suppressing the *alr* mutation present in TKL 10. In these crosses transfer of *alr⁺* is very low as a result of the distance of the *alr* locus from the origins of both the Hfr strains (as shown in Fig. 1). The transfer of *msuA⁻* from strains KMBL 171 and B 9 is instead found, demonstrating its high linkage to *pyrF*.

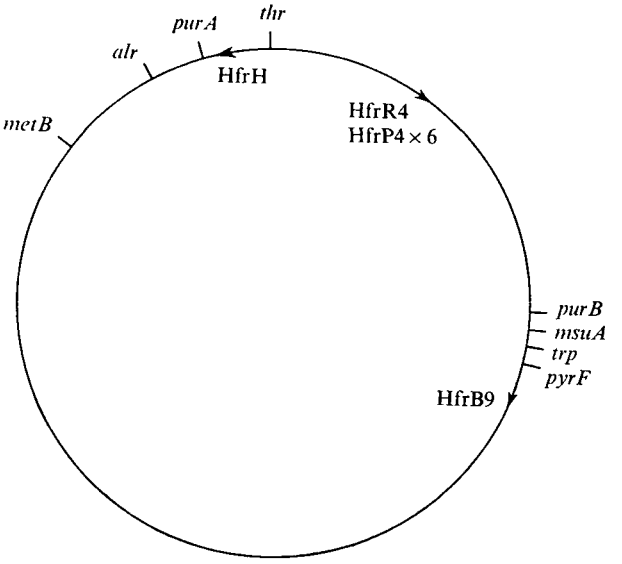

Fig. 1. Linkage map of *E. coli*, adapted from Taylor (1970), indicating the location of points of origin of Hfr strains and markers used.

L-alanine at 42 °C. Growth on L-alanine at 42 °C only occurs when *alr*$^+$ has also been introduced.

The presence of two independent mutations can also be demonstrated the other way round. D-Alanine-independent (*Alr*$^+$) transductants are still unable to grow on L-alanine, but this ability may be recovered as a result of further transduction.

(iii) *Conjugation with different Hfr strains*

To determine the position of the *alr* locus, KMBL171 (HfrH) was mated with TKL10 and different types of recombinants were selected. From its relative frequency of transmission, *alr* appeared to be located near *pyrF* and this seemed to be confirmed by the high linkage of *alr* to *pyrF* (Table 3; Fig. 1). For further confirmation, another Hfr strain (HfrB9) was used which transfers *pyrF* as an early marker and in the opposite direction to HfrH. As Table 3 shows, in this cross *alr* had a high linkage with *trp* and *pyrF*. The sequence of these genes is *pyrF–trp–alr*, assuming that the Alr$^+$Trp$^-$PyrF$^+$ recombinant class is absent because it can only be generated by a multiple crossover event.

The high linkage with *pyrF* indicated the possibility of cotransduction of the two markers. However, as described in the next section, although a high proportion (83 %) of transductants to Alr$^+$ (using KMBL171 as donor strain) also inherited the donor *pyrF*$^+$ marker, when an *alr pyrF*$^+$ derivative of TKL10 was used as donor and *pyrF*$^+$ selected, none of the transductants received the *alr* allele. This suggested that *alr* and *pyrF* are not in fact closely linked, but that the locus cotransducible with *pyrF* is a suppressor of the Alr$^-$ phenotype. This was supported by the further finding that the Hfr strain GIA26 (Table 1), when used as a trans-

Table 4. *Analysis of recombinants from a cross HfrR4 × TKL10*

Selected marker	No. of recombinants tested	Unselected markers	No.
alr$^+$	148	*purA*$^-$ *thr*$^+$	55 (37%)
		purA$^-$ *thr*$^-$	39 (26%)
		purA$^+$ *thr*$^-$	41 (28%)
		purA$^+$ *thr*$^+$	13 (9%)

Strain H787 (HfrR4) was mated with TKL10 and Alr$^+$ recombinants were selected on minimal medium at 42 °C, with streptomycin as counterselective agent. The degree of transfer of the *msuA* locus of H787 in the cross is very low as a result of the distance of *msuA* from the origin of the Hfr strain. Therefore most of the Alr$^+$ recombinants will have received the *alr*$^+$ allele.

ductional donor, did not cotransduce the Alr$^+$ phenotype with *pyrF*. Presumably the suppressor, for which the new symbol *msuA* is proposed, is present in the Hfr strains used, but absent from GIA 26.

The position of the *alr* locus was reassessed by way of conjugal crosses. Since GIA26 transferred *alr*$^+$ with a frequency suggesting that markers around 80 min on the conventional map (Taylor, 1970) would be suitable for more accurate location, a *purA metB* HfrR4 strain (H787) was used, which transfers *pyrF* as a late marker (see Fig. 1). Among *alr*$^+$ recombinants selected, linkage of *alr* with *purA* was much more frequent (63%) than with *metB* (21%). This suggests that *alr* is located on the same side of *metB* as the *purA* marker. Confirmation comes from the distribution of *purA* and *thr* among *alr*$^+$ recombinants (Table 4). The low frequency of the *alr*$^+$*purA*$^+$*thr*$^+$ class indicates the requirement for four crossing-over events, and suggests the order *thr–pur–alr*. The *purA* marker has been mapped by Verhoef & de Haan (1966) at about 3·5 min from *thr* on the conventional map. Because the linkage of *alr* with *purA* (63%) is about the same as the linkage of *purA* with *thr* (65%), *alr* may be supposed to be located at about 83 min. Markers with which *alr* might be cotransducible were not available.

(iv) *Cotransduction of* msuA *with other genes*

For identifying the suppressor gene near *pyrF*, relevant genes to test for cotransduction with *msuA* are *pyrF*, *trp* and *purB*. A very low frequency of cotransduction with *purB* was found (Table 5). Cotransduction with *pyrF* and *trp* was found as well, indicating a high linkage to both markers. The gene order *pyrF–trp–msuA* is confirmed by the three-point cross.

4. DISCUSSION

From the experiments reported above it can be concluded that TKL10 has a mutation in the structural gene for D-alanine:L-alanine racemase which impairs the *in vivo* activity of the enzyme at 42 °C. One must assume that the *in vivo* activity at the permissive temperature is sufficient. In the assay at 28 °C, however,

Table 5. *Cotransduction of* msuA

Donor	Recipient	Phenotype selected	No. of transductants tested	Unselected marker(s)	No.
H731	TKL10	Alr$^+$	189	*purB*$^-$	1
alr$^+$ *msuA*$^-$ *purB*$^-$	*alr*$^-$ *msuA*$^+$ *purB*$^+$	(*alr*$^+$ + *msuA*$^-$)			
KMBL171	TKL10	Alr$^+$	30	*trp*$^+$ *pyrF*$^+$	26
alr$^+$ *msuA*$^-$ *trp*$^+$ *pyrF*$^+$	*alr*$^-$ *msuA*$^+$ *trp*$^-$ *pyrF*$^-$	(*alr*$^+$ + *msuA*$^-$)		*trp*$^+$ *pyrF*$^-$	2
				trp$^-$ *pyrF*$^-$	2
				trp$^-$ *pyrF*$^+$	0

P 1 lysates on the donor strains were added to TKL10 and Alr$^+$ recombinants were selected; these could be *alr*$^+$ or *msuA*$^-$, the allele *msuA*$^-$ suppressing the *alr*$^-$ allele in TKL10. For reasons mentioned in the text, Alr$^+$ recombinants which have co-inherited markers in the *purB–trp–pyrF* region are supposed to carry *msuA*$^-$.

the level of alanine racemase activity was not significantly higher than at 42 °C. Such an absence of enzyme activity at the non-restrictive temperature has been found in other types of temperature-sensitive mutants as well (Eidlic & Neidhardt, 1965; Böck & Neidhardt, 1966; Yaniv & Gros, 1969). Neidhardt (1966) has ascribed the phenomenon to an increased sensitivity of the enzyme for the *in vitro* conditions, accompanying the changed steric conformation of the enzyme which impairs its activity *in vivo* at 42 °C.

The genetic symbol *alr* has been chosen to indicate a mutation affecting the structure of this enzyme, in conformity with the nomenclature in *Bacillus subtilis* (Berberich *et al.* 1968). The *alr* gene has been mapped at about 83 min on the current map (Taylor, 1970). So far, no other cell-wall genes have been reported in this region.

During these experiments a suppressor gene *msuA* was identified. The gene *msuA* must be very close to the loci of the amber and ochre suppressors *supC*, *supO* and *supF* (Taylor, 1970) but, since a thermosensitive mutation is suppressed, a missense suppressor must be involved. It seems probable that the suppressing allele was present in the common parent of the Hfr strains of Hayes, Reeves and Broda.

As it stands, the possibility that the gene called *msuA* is in actual fact a second copy of the *alr* gene, translocated to the *pyrF–trp* region, cannot be ruled out. Among Alr$^+$ transductants, the *msuA*$^-$ type exceeded the *alr*$^+$ type, as can be deduced from the very high degree of cotransduction with *pyrF* (Table 5). The apparent superior viability of *msuA*$^-$ cells can perhaps be explained by advantageous effects of suppression of other hypothetical deleterious mutations.

In the literature, some other mutants which require D-alanine for growth have been described. Matsuzawa *et al.* (1969) have described a strain ST 640 in which lysis is prevented by a high concentration of D-alanine in the medium (1000 μg/ml, instead of 40 μg/ml in TKL 10). Since the mutation in ST 640 is located near *leu*, it cannot involve the gene for alanine racemase, as suggested by the authors. In fact, a deficiency in the enzyme responsible for the dimerization of D-alanine was found (Lugtenberg & van Schijndel-van Dam, 1973), while the racemase was normal. The mutant, *sud-25* (Mangiarotti, Apirion & Schlessinger, 1966), is interesting in that it forms long filaments (eventually resulting in lethality), which may be prevented by the addition of either 20 % sucrose or D-alanine. The authors do not mention the concentration of alanine needed, and whether *sud-25* is allelic to *alr* or to the mutation in ST 640 is not known.

The question may be raised whether the *alr*$^+$ function has a direct relationship to cell-wall synthesis. D-Alanine plays a role in cell-wall synthesis in its dipeptide form, D-alanyl-D-alanine (Strominger, 1962). Every cell needs the dipeptide, but possibly a basal level of D-alanine suffices for its synthesis. With respect to the inducibility of the enzyme, *E. coli* seems to differ from *Bacillus subtilis* (Berberich *et al.* 1968). Just as in *Pseudomonas putida* (Rosso *et al.* 1969), the inducibility of alanine racemase in *E. coli* suggests that its primary function in the cell is to convert L-alanine to pyruvate via D-alanine. This is in agreement with the isolated

site of the *alr* locus with respect to several genes directly involved in the synthesis of the pentapeptide precursor of the mucopeptide layer of the cell wall (Wijsman, 1972), among which is the gene concerned with the synthesis of D-alanyl-D-alanine.

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REFERENCES

Berberich, R., Kaback, M. & Freese, E. (1968). Amino acids as inducers of L-alanine dehydrogenase in *Bacillus subtilis*. *Journal of Biological Chemistry* **243**, 1006–1011.

Böck, A. & Neidhardt, F. C. (1966). Isolation of a mutant of *Escherichia coli* with a temperature-sensitive fructose-1,6-diphosphate aldolase activity. *Journal of Bacteriology* **92**, 464–469.

Broda, P. (1967). The formation of Hfr strains in *Escherichia coli* K 12. *Genetical Research, Cambridge* **9**, 35–47.

Eidlic, L. & Neidhardt, F. C. (1965). Protein and nucleic acid synthesis in two mutants of *Escherichia coli* with temperature-sensitive aminoacyl ribonucleic acid synthetases. *Journal of Bacteriology* **89**, 706–711.

Lennox, E. S. (1955). Transduction of linked genetic characters of the host by bacteriophage P 1. *Virology* **1**, 190–206.

Lugtenberg, E. J. J. & van Schijndel-van Dam, A. (1973). A temperature sensitive mutant of *Escherichia coli* K12 with an impaired D-alanine : D-alanine ligase. *Journal of Bacteriology*, (in the Press).

Mangiarotti, G., Apirion, D. & Schlessinger, D. (1966). Selection of sucrose dependent *Escherichia coli* to obtain envelope mutants and fragile cultures. *Science, New York* **153**, 892–894.

Matsuzawa, H., Matsuhashi, M., Oka, A. & Sugino, Y. (1969). Genetic and biochemical studies on cell wall peptidoglycan synthesis in *Escherichia coli* K-12. *Biochemical and Biophysical Research Communications* **36**, 682–689.

Neidhardt, F. C. (1966). Roles of amino acid activating enzymes in cellular physiology. *Bacteriological Reviews* **30**, 701–718.

Okada, T., Yanagisawa, K. & Ryan, F. J. (1960). Elective production of thymineless mutants. *Nature, London* **188**, 340–341.

Rosso, G., Takashima, K. & Adams, E. (1969). Coenzyme content of purified alanine racemase from *Pseudomonas*. *Biochemical and Biophysical Research Communications* **34**, 134–140.

Strominger, J. L. (1962). Biosynthesis of bacterial cell walls. In I. C. Gunsalus and R. Y. Stanier (eds.), *The Bacteria*. Vol. III. *Biosynthesis*, pp. 413–470. New York and London: Academic Press.

Taylor, A. L. (1970). Current linkage map of *Escherichia coli*. *Bacteriological Reviews* **34**, 155–175.

Verhoef, C. & De Haan, P. G. (1966). Genetic recombination in *Escherichia coli*. I. Relation between linkage of unselected markers and map distance. *Mutation Research* **3**, 101–110.

Waddell, W. J. (1956). A simple ultraviolet spectrophotometric method for the determination of protein. *Journal of Laboratory and Clinical Medicine* **48**, 311–314.

Wijsman, H. J. W. (1970). Een genetische studie over de celwandsynthese bij *Escherichia coli*. Ph.D. Thesis, University of Amsterdam.

Wijsman, H. J. W. (1972). A genetic map of several mutations affecting the mucopeptide layer of *Escherichia coli*. *Genetical Research Cambridge* **20**, 65–74.

Yaniv, M. & Gros, F. (1969). Studies on valyl-tRNA synthetase and $tRNA^{val}$ from *Escherichia coli*. III. Valyl-tRNA synthetases from thermosensitive mutants of *Escherichia coli*. *Journal of Molecular Biology* **44**, 31–45.