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A Mutation in *Salmonella typhimurium* Imparting Conditional Resistance to 5-Fluorouracil and a Bioenergetic Defect: Mapping of *cad*

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Summary. The position of the genetic locus allelic with the *cad-2* mutation has been located between units 14 and 15 of the linkage map of *S. typhimurium*. Fine structure mapping established the gene order as *cad flrB nag*. The genetic evidence coupled with biochemical evidence indicates that this *cad* locus is homologous to the *ubiF* gene of *Escherichia coli*.

Introduction

As a function of a continuing study on the regulation of pyrimidine metabolism in *S. typhimurium*, a novel mutant was recently isolated which was dependent upon an exogenous supply of carbamylaspartate (CAA; an intermediate of the *pyr* biosynthetic pathway) for resistance to 5-fluorouracil (5-FU). Particulars pertaining to the isolation and characterization of the mutant will comprise a separate report but various salient characteristics are summarized below.

The mutant (strain KR42; refer to Table 1) appeared to be impaired bioenergetically as evidenced by the following: a) in both glucose minimal medium and complex medium, the growth rate and growth yield were reduced; b) the mutant failed to grow when citrate or succinate was the sole carbon and energy source; c) the activity of the *pyr* enzyme dihydroorotate dehydrogenase (DHOdehase) was barely measurable by a conventional assay procedure which requires that an extract has a functional ubiquinone and cytochrome electron acceptor system for the oxidation of dihydroorotate (Taylor and Taylor 1964). Using the alternative assay procedure (Karibian 1973) which utilizes dichlorophenolindophenol as an exogenous electron acceptor, DHOdehase activity could be readily measured in extracts from the mutant. On the basis of this latter result and the aforementioned growth characteristics, it was inferred that the mutation (arbitrarily designated *cad*) resulted in an alteration of the operation of the electron transport chain.

To further characterize the mutant, experiments were initiated to map the *cad* mutation. The main purpose of the mapping study was to gain further information which could prove useful in identifying the biochemical basis for CAA-dependent 5-FU resistance and the associated pleiotropy.

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Materials and Methods

Organisms

The bacterial strains used in this study are listed in Table 1. Bacteriophages HT7 (a high frequency transducing, integration defective derivative of phage P22) and P1*kc* were obtained from J.L. Ingraham.

Media and Conditions for Growth

The basic composition of the two minimal media employed, TF and A, has been described previously (Kelln et al. 1975). The carbon sources used with TF were 0.2% glucose (TFG medium), 0.3% citrate (TFC medium), 0.2% galactose, 0.3% glucosamine, or 0.3% *N*-acetylglucosamine; with medium A, only glucose at a final concentration of 0.2% (AG medium) was used. Supplements, when required, were added at the following concentrations: amino acids, 50 μg/ml; lipoate, 2 μg/ml; 5,5,5-trifluoro-*DL*-leucine, 1 mg/ml (used solely with AG medium); 5-fluorouracil, 5 μg/ml; carbamylaspartate, 50 μg/ml; and ethyleneglycol-*bis*-(β-amino-ethyl ether)-*N,N'*-tetraacetate (EGTA), 10 mM. The composition of the complex medium (LC) has been described elsewhere (Kelln et al. 1975) as has the composition of tetrazolium indicator medium (Ohlsson et al. 1968). Solid media contained 15 g agar/l.

The incubation temperature was 37° C with the exception of P1*kc*-mediated transductions where the plates were incubated at 30° C. Liquid cultures were grown on a gyrorotary shaker operating at 250 rpm.

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Indicator Plates for the Detection of Leucine Overproduction

Plates were prepared essentially as described by O'Donovan and Gerhart (1972). Exponentially growing cells of strain L39 were added to TFG medium to give a final cell number of 2×10^7/ml. Plates were stored at 4° C and used within 96 h following preparation.

Mutagenesis and Isolation of Mutants

Mutagenesis of strain SGSC9 with ethyl methanesulphonate was carried out according to the procedure of Hayashi et al. (1964). The mutagenized culture was subjected to penicillin counterselection (10,000 units pencillin G/ml) for 2 h in TF medium with *N*-acetylglucosamine as carbon source. Following the counterselection and washing of the surviving cells, samples were plated on tetrazolium indicator plates containing 1% *N*-acetylglucosamine for purposes of indicating *nag* derivatives.

The isolation of the *galE* derivative (strain KR53) of strain KR42 was accomplished using a previously described procedure (Beck et al. 1972).

Genetic Techniques

Transductions mediated by phage HT7 were done as described by Ely et al. (1974) and those mediated by P1*kc* as described by Kelln et al. (1975).

The transfer of the F'152 episome was accomplished by directly plating the donor and recipient on a selective medium. Resulting transconjugants were purified by streaking on a selective medium.

Crosses with Hfr strains were achieved by either the direct plating method (see above) or by the procedure described by Kelln et al. (1975) for interrupted mating experiments.

Chemicals

All chemicals were reagent grade and most were purchased from Sigma Chemical Co. The 5,5,5-trifluoro-*DL*-leucine was obtained from Aldrich Chemical Co., Inc.

Results and Discussion

Selection of Cit$^+$ Recombinants

Strain KR42 was incapable of utilizing citrate as sole carbon source (phenotypically Cit$^-$) due to the presence of the *cad* mutation. In transducing strain KR42 for utilization of citrate (Cit$^+$ phenotype), the transductants simultaneously lost the property of CAA-dependent 5-FU resistance. This latter aspect was also observed with Cit$^+$ revertants of strain KR42. Thus, acquisition of the Cit$^+$ phenotype reflected a genotypic change to *cad*$^+$, and accordingly, introduction of *cad*$^+$ was readily selected by plating on TFC medium.

Conjugation Experiments

Initially various Hfr strains (refer to Table 1 and Fig. 1) were assessed by the direct plating method

Table 1. Bacterial strains

Strain	Relevant genotype[a]	Source
S. typhimurium LT2		
CV117	*ara-9 gal-205 leuS2*	J.M. Calvo
TR3684	*hisO1242 hisDG580 nag-1*	J.M. Calvo
KP1274	*galE met val*	J. Neuhard
JL84	Hfr A, *met B406 hisD23 gal-50*	J.L. Ingraham
L39	*leuBCD39*	K.E. Sanderson
SU436	Hfr B3, *hisD23 gal-50*	K.E. Sanderson
SA534	Hfr K4, *serA13 rfa-3058*	K.D. Sanderson
SA828	Hfr K25, *serA15 rfx*	K.E. Sanderson
SA965	Hfr K17, *leuBCD39 ara-7*	K.E. Sanderson
SA342	*ara-9 lip-2*	K.E. Sanderson
SGSC9[b]	*ara-9 flrB1*	K.E. Sanderson
KR42	*usp-3 cad-2*	Own collection
KR53	*usp-3 cad-2 galE*	This study
KR58[b]	*ara-9 flrB1 nag*	This study
E. coli K-12		
PL225/F'152	*recA str thi Δ(gal-nadA) λ$^-$/F$^+$ (gal$^+$-lip$^+$)*	E. Ohtsubo

[a] The symbol, *usp*, denoting the gene(s) governing permeability to ureidosuccinate (i.e. carbamylaspartate) was introduced by Syvanen and Roth (1973) and has been approved for use by K.E. Sanderson of the Salmonella Genetic Stock Centre. The gene symbol *cad* is our arbitrary designation for denoting genotype with respect to CAA-dependent 5-FU resistance

[b] Strain SGSC9, the parental strain of KR58, was described as harboring the *gal-205* mutation. However, we found the strain to be Gal$^+$ and thus, the *gal-205* mutation has not been included in the description of the genotype

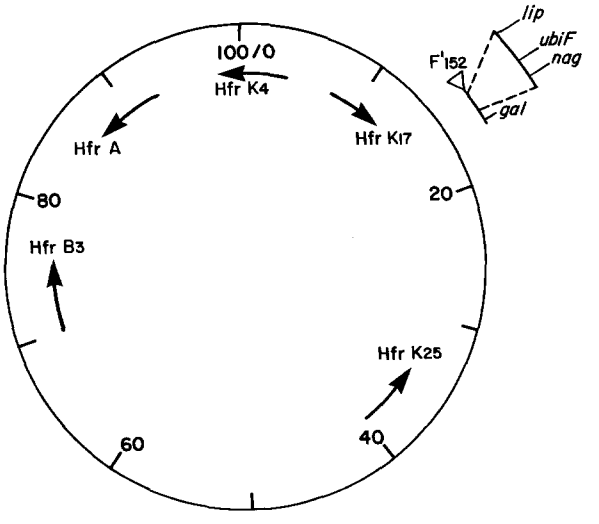

Fig. 1. Circular linkage map of *S. typhimurium* drawn to 100 units according to the recent presentation of Sanderson and Hartman (1978). The closed arrows inside the circle indicate the origin and direction of transfer of the Hfr strains used; the open arrow at the outer edge of the circle represents the *E. coli* K-12 episome (F'152) used, with the location of relevant genes on the episome indicated

for their efficiency in transferring cad^+ to strain KR42. The best donor proved to be strain SA534 (Hfr K4) and it was chosen for time of entry experiments. However, a difficulty was encountered whereby the liquid mating procedure yielded few Cit^+ recombinants, indicative of a poor efficiency of transfer. Beck et al. (1977) have reported encountering a similar difficulty which could be overcome by the introduction of a *galE* mutation into the recipient. Thus, a *galE* derivative of strain KR42 was constructed, namely strain KR53.

With strain KR53 an improved efficiency of transfer was achieved and since the strain was Gal^-, selection for transfer of an additional marker was also possible. In an experiment designed to select for the transfer of $galE^+$ from strain SA534, we observed that over 90% of the recombinants had simultaneously inherited cad^+. This result indicated that *cad* was close to *galE*.

F-Prime Complementation Study

To further localize the position of *cad*, F'152 (see Fig. 1) was transferred to strain KR53 by selecting for the transfer of $galE^+$. All Gal^+ transconjugants were also Cit^+ and sensitive to 5-FU in the presence of CAA. The F'152 episome encompasses the region of *fep* to *gal* (Ohtsubo and Hsu 1978) and thus, the gene complementing the *cad* mutation was located within those limits.

Transductional Analyses: Establishing Linkage

Phage P1*kc* was propagated on strain KP1274 and the lysate was used for transduction of strain KR53 to Gal^+ by selecting for $galE^+$ interallelic recombinants. In the single transduction performed, thirty-five Gal^+ transductants were obtained; all were Cit^- and resistant to 5-FU in the presence of CAA. Thus, if *cad* were linked to *galE*, the linkage was less than 3% with P1*kc* as transductional vector.

Linkage of *cad* with other genes in this region of the chromosome was also tested. In transductions using phage HT7, linkage of *cad* with *leuS*, *flrB* and *nag* was observed (Table 2). Extremely close linkage of *cad* and *flrB* was evident and close linkage of *cad* and *nag* was also apparent, consistent with the observation that *flrB* and *nag* are 75% cotransducible by phage P22 (J.M. Calvo, personal communication).

Table 2. Transductions using various donors

Donor	Recipient	Transductants[a]			% Cotransduction
		Selected marker	Unselected marker	Unselected/selected	
CV117	KR42	*cad*$^+$	*leuS2*	3/300	1.0
TR3684	KR42	*cad*$^+$	*nag-1*	451/561	80
SA342	KR42	*cad*$^+$	*lip-2*	0/504	< 0.2
SGSC9	KR42	*cad*$^+$	*flrB1*	587/594	98

[a] The cad^+ transductants were selected by plating samples of the transductional mixtures on TFC medium or TFC medium containing lipoate when strain SA342 was used as donor. The introduction of the *leuS2* or *flrB1* allele was scored by spotting the transductants on both leucine indicator plates and TFC medium containing 1 mg 5,5,5-trifluoro-*DL*-leucine/ml. Those transductants receiving the *nag-1* mutation were detected by their inability to use *N*-acetylglucosamine as sole carbon source. Testing for the presence of *lip-2* was done by spotting on TFC medium

Transductional Analyses: Establishing Gene Order

With linkage established, the aspect remaining was the ordering of *cad* with respect to *flrB* and *nag*. In order to accomplish the above, it was necessary to construct an appropriate donor strain having both a *flrB* and a *nag* mutation. The parental strain (SGSC9; *flrB1*) was mutagenized and then subjected to a counterselection procedure. Surviving cells unable to ferment *N*-acetylglucosamine (Nag^-) were detected as red colonies on the indicator plates. The non-fermenting clones were further tested by spotting onto medium with either *N*-acetylglucosamine or glucosamine (Gcn) as sole carbon source. This spot testing was done in order to identify isolates of the phenotype Nag^-, glucosamine utilizing (Gcn^+). The rationale for desiring such isolates was based on the observation that strain TR3684 (used as donor in establishing linkage of *cad* and *nag*) was phenotypically Nag^- Gcn^+. Isolates having a Nag^-Gcn^+ phenotype were then checked for having retained the *flrB* mutation.

Following the above assessment of phenotype, it was then necessary to determine whether the Nag^- character was cotransducible with *cad* as was the case with strain TR3684 as donor. With one isolate, strain KR58, a cotransduction frequency of 73% for Cit^+ (cad^+) and Nag^- (*nag*) was observed (Table 3A) and therefore, it was considered an appropriate donor strain for the three-factor cross.

The results of the three-factor cross are presented in Table 3B. The rationale for the mapping method is described in the legend to Fig. 2. Based on this rationale, consideration of the low frequency of occurrence of the *nag* mutation among cad^+flrB^+ transductants indicates that gene order one is correct.

A summary of the results from phage HT7-mediated transductions is presented in Fig. 3. Friedberg et al. (1974) reported that *flrB* and *leuS* were not cotransducible in phage P22-mediated transductions.

Table 3. Transductions using strain KR58 as donor

A. Testing for linkage of *cad* and *nag*

Recipient	Transductants[a]			% Cotransduction
	Selected marker	Unselected marker	Unselected/selected	
KR42	*cad*$^+$	*nad*	147/202	73

[a] The *cad*$^+$ transductants were obtained and scored for *nag* as described in the legend to Table 2

B. Three-factor cross

Recipient	No. of transductants of specific genotype[a]		
	cad$^+$	*cad*$^+$ *flrB*$^+$	*cad*$^+$ *flrB*$^+$ *nag*
KR42	3838	161	17

[a] The *cad*$^+$ transductants were selected on TFC medium containing EGTA. The EGTA was added to minimize lysogeny of transductants (Kleckner et al. 1975) which could complicate the subsequent testing for leucine overproduction (Kelln et al. 1975). Those transductants of the genotype *cad*$^+$ *flrB*$^+$ were determined through their lack of excretion of leucine as evidenced on indicator plates and their sensitivity to 5,5,5-trifluoro-*DL*-leucine. These transductants were then tested for the ability to use *N*-acetylglucosamine as sole carbon source

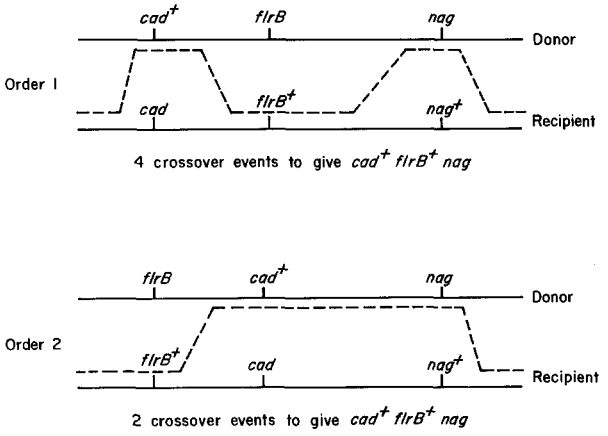

Fig. 2. Three-factor mapping method. When order 1 pertains, four crossover events are required to give *cad*$^+$ *flrB*$^+$ *nag* transcuctants; thus, their occurrence among total *cad*$^+$ *flrB*$^+$ transductants will be infrequent (10 to 25%). In a situation where order 2 applies, only two crossover events are necessary to give *cad*$^+$ *flrB*$^+$ *nag* transductants and their percentage among total *cad*$^+$ *flrB*$^+$ transductants will be high (50 to 75%)

While a similar experiment was not attempted here, we did observe cotransduction of *cad* and *leuS* by a P22 phage (HT7), albeit at a low frequency (1%). Interpretation of the three-factor cross placed *cad* counterclockwise of *flrB* and thus, observing linkage of *cad* and *leuS* at low frequency is not an unreasonable result.

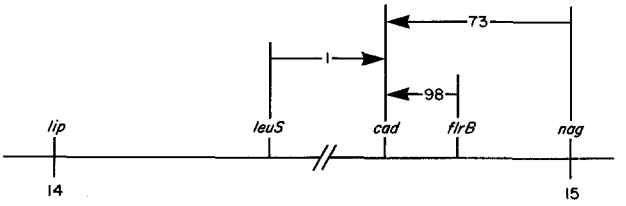

Fig. 3. Segment of the genetic linkage map of *S. typhimurium* encompassing units 14 to 15: based on data presented in this report and on information included in the article by Sanderson and Hartman (1978). The arrows point in the direction of the selected donor gene and the numbers between genes indicate the cotransduction frequencies in phage P22 (specifically HT7)-mediated transduction. The value for cotransduction of *cad* and *nag* was from the data presented in Table 3A; other values are from data given in Table 2. The genetic symbol *cad* is used here since identification of *cad* as *ubiF* is only tentative

Further Considerations Following Localization of the cad Mutation

Though several phenotypic properties imparted by the *cad* mutation (data to be presented elsewhere) and the localization of *cad* on the *S. typhimurium* chromosome had been ascertained, the true nature of the mutation remained unknown. The inability of strain KR42 (and KR53) to use citrate or succinate as sole carbon and energy source in association with the poor growth rate and yield indicated a bioenergetic impairment. The lack of endogenous electron accepting capacity shown by cell extracts suggested that the impairment resided with the operation of the electron transport chain.

Localizing the *cad* mutation on the *S. typhimurium* chromosome allowed for a comparison to the linkage map of the *E. coli* K-12 chromosome (Bachmann et al. 1976), the gene composition of the relevant region of the chromosome having been more extensively defined in *E. coli*. From such a comparison, we acknowledged that a mutation in *S. typhimurium* occurring at any of three loci homologous with *sucA, sucB* or *ubiF* of *E. coli* would result in a reduced capacity for utilization of citrate or succinate as carbon and energy source. Langley and Guest (1974) reported that *sucA* mutants of *S. typhimurium* required succinate for growth on glucose minimal medium, a property not characteristic of the *cad* mutation. Thus, if *cad* were equivalent to *sucA,* the *cad* mutation was "leaky". However, several lines of evidence suggested that *cad* and *suc* mutations were not equivalent. Addition of succinate (final concentration, 0.2 mM) to the medium did not stimulate the growth of strain KR42, contrary to results expected if the strain was a "leaky" *sucA* or *sucB* mutant. Also *sucA* and *sucB* are cotransducible with *gal* in *E. coli* using phage P1*kc* and, assuming a similar genetic organization

of the *S. typhimurium* chromosome, cotransduction of *cad* and *gal* should have occurred but did not. As a final point, *sucA* or *sucB* mutations would not be expected to result in reduced endogenous electron accpeting capacity and this aspect was clearly evident from the DHOdehase assays done with strain KR42 cell extracts.

The other gene for consideration was *ubiF* which, in *E. coli,* is linked to *nag* in transduction with phage P1*kc* (Holmes and Russell 1972) and furthermore, is not cotransducible with *gal* (Young et al. 1971). Thus, linkage aspects for *cad* in *S. typhimurium* and for *ubiF* in *E. coli* are similar. Also, since ubiquinone is required as an electron acceptor for DHOdehase in aerobic growth conditions (Newton et al. 1971), a deficiency in ubiquinone biosynthesis would explain the reduced activity of DHOdehase observed in strain KR42 cell extracts when assayed by a method necessitating an endogenous electron accepting capacity. Thus, characterization, both genetic and biochemical, suggests that strains KR42 and KR53 are *ubiF* mutants and accordingly the *cad-2* mutation would be more aptly described as a *ubiF* mutation. If the foregoing is correct, the *ubiF* gene of *S. typhimurium* is located on the chromosome at a position analogous to that of the *E. coli ubiF* gene. However, the means by which a *ubiF* mutation confers CAA-dependent 5-FU resistance remains unexplained and clarification must await further study.

Experiments are in progress to determine whether or not the *cad* mutants are deficient in ubiquinone biosynthesis.

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