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Tandem Genetic Duplications in *Salmonella typhimurium*: Amplification of the Histidine Operon

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Salmonella strains harboring tandem duplications of chromosomal segments including the histidine operon may be selected from populations of *hisO* promoter-like mutants. The twofold increase in gene dosage of the histidine operon caused by tandem duplication provides resistance to concentrations of the histidine analogue 3-amino-1,2,4-triazole that inhibit the growth of haploid *hisO* mutants. Several properties of AT‡-resistant mutants indicate that they harbor tandem chromosomal duplications: (i) the AT-resistant phenotype of these strains is genetically unstable. Such instability is dependent upon a functional recombination system. (ii) AT-resistant mutants express approximately twice the levels of *his* enzymes as parental strains. (iii) Genetic tests indicate that AT-resistant mutants are merodiploid for large segments (up to 26%) of their genome. (iv) For certain isolates, the merodiploid and AT-resistant nature of these strains are properties cotransducible with a distant chromosomal marker unrelated to the *his* operon. We interpret these results as indicating cotransduction of the join-point of a tandem duplication with this distant marker.

The spontaneous frequency of tandem *his* duplications is remarkably high ($6{\cdot}2 \times 10^{-5}$ per cell). This frequency is more than 6000-fold reduced in *recA*$^-$ genetic backgrounds. Two major types of tandem *his* duplications are repeatedly isolated, having an identical ~13% or ~22% of their genome duplicated, respectively. When duplication-containing strains are grown under conditions that select for resistance to increased AT concentrations, clones harboring additional tandem copies (amplification) of the *his* operon are obtained. The role which such gene amplification may play in bacterial adaptation is discussed.

1. Introduction

Duplication of genetic material has been suggested to play an important role in molecular evolution (Hegeman & Rosenberg, 1970; Ohno, 1970). Duplications may increase the dosage of a required allele (gene amplification) or supply the redundant DNA necessary for genetic divergence. Within the past several years, methods have been developed for the detection and analysis of tandem genetic duplications in bacteria and their phages (see review by Anderson & Roth, 1977). The results of these studies suggest that tandem duplications are remarkably frequent in both *Escherichia*

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‡ Abbreviation used: AT, 3-amino-1,2,4-triazole.

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coli and *Salmonella typhimurium* (Langridge, 1969; Miller & Roth, 1971; Hill & Combriato, 1973; Straus, 1974; Straus & Hoffmann, 1975; Straus & Straus, 1976; Anderson *et al.*, 1976), and that such duplications may play an important role in bacterial adaptation (Novick & Horiuchi, 1961; Horiuchi *et al.*, 1963; Rigby *et al.*, 1974; Hashimoto & Rownd, 1975).

Two different selections for detecting tandem duplications of the histidine operon in *Salmonella* have previously been described. One is based upon the ability of duplication-containing strains to be made heterozygous for complementing *his* mutations (Anderson *et al.*, 1976). The second is based upon the ability of tandem duplications to fuse histidine genes to "foreign" promoter elements (Anderson & Roth, 1978). However, each of these selections suffers inadequacies that limit its usefulness for a general study of the duplication process. The selection based upon detection of complementing heterozygotes involves transduction and can only be performed in recombination-proficient (*rec*$^+$) backgrounds. The selection based upon fusion of *his* genes to different promoter elements severely limits the chromosomal location of detectable duplication end-points.

In this paper we describe a selection for tandem duplications of the *his* operon that is more generally useful in studying the duplication process. It is based upon a gene dosage effect exhibited by certain *his* merodiploids. Strains harboring tandem duplications are selected because of their doubling of *his* enzyme levels. We have utilized this selection to investigate the size and frequency of spontaneously occurring tandem duplications of the *his* operon and the involvement of the *Salmonella* recombination system in this process.

2. Materials and Methods

(a) *Media and growth conditions*

Vogel & Bonner's (1956) E medium containing 2% glucose was used as minimal medium. When required, this medium was supplemented with 0·1 mM-histidine, 4 mM-serine, 0·4 mM each of adenine, guanine and arginine, 0·05 mM-thiamin, and other amino acids (approx. 0·3 mM). DL-amino acids were often used, but the concentrations given are for the L-isomer. Adenine plus thiamin were added to all media that contained 3-amino-1,2,4-triazole. Tetracycline was added to complex media at 25 μg/ml and to minimal media at 10 μg/ml. When desired as a sole carbon source, D-sorbitol, D-trehalose, sodium gluconate, or glucuronic acid were added at 0·2% (w/v) to E medium from which glucose and citrate had been omitted. Difco nutrient broth (0·8%) containing 0·5% NaCl was used as complex medium. Solid media contained 1·5% Difco agar. All incubations were performed at 37°C. Liquid cultures were aerated by gyratory shaking.

(b) *Bacterial strains*

The genotypes and sources of selected strains used in this study are shown in Table 1. All gene designations are those of the revised *Salmonella* genetic nomenclature (Sanderson & Hartman, 1978). Strains with "TT" designations are those in our collection that either contain or are descended from a strain containing a copy of the translocatable tetracycline-resistance determinant Tn*10* (Kleckner *et al.*, 1975). All strains are derivatives of *S. typhimurium* strain LT2.

Strain TR4179 (*hisO2355 srl-201*) was derived by transducing the mutation *hisO2355* from strain TR3578 into strain TR4178 (*hisΔ203 srl-201*) (Anderson & Roth, 1978), selecting His$^+$. The mutation *srl-201* is approximately 50% linked to *recA1* by bacteriophage P22-mediated generalized transduction. The close proximity of these 2 loci in *E. coli* has been described (McEntee, 1976; A. J. Clark, personal communication). Strain TR4255 (*hisO2355 srl-201 recA1 strA*) was derived by mating donor strain TR2246

TABLE 1

List of strains

Strain	Genotype	Source
TR3578	*hisO2355*	P. E. Hartman
TR4179	*hisO2355 srl-201*	This paper
TR4255	*hisO2355 srl-201 recA1 strA*	This paper
TR2951	*hisΔ63 recA1 strA*	Anderson & Roth (1978)
TR5368	*hisO2355 srl*$^+$ *recA*$^+$	TR2951 × TR4179
TR5369	*hisO2355 srl*$^+$ *recA1*	TR2951 × TR4179
TT2279	*hisΔ515(gnd*$^-$*rfb*$^-$*) met-521 eda-100*/F′$_{400}$ *hisO2355 gnd*$^+$*rfb*$^+$*zzf-20*::Tn*10*	This paper
TT2280	*hisO2355 recA1*/F′$_{400}$ *hisO2355 zzf-20*::Tn*10*	TT2279 × TR5369
TT1984	*hisG8575*::Tn*10 hisO2355 srl-201*	This paper
TT1985	*hisD8578*::Tn*10 hisO2355 srl-201*	This paper
TT1986	*hisC8579*::Tn*10 hisO2355 srl-201*	This paper
TT1987	*hisB8686*::Tn*10 hisO2355 srl-201*	This paper
TT1988	*hisH8576*::Tn*10 hisO2355 srl-201*	This paper
TT1989	*hisA8676*::Tn*10 hisO2355 srl-201*	This paper
TT1990	*hisF8539*::Tn*10 hisO2355 srl-201*	This paper
TT1991	*hisI9537*::Tn*10 hisO2355 srl-201*	This paper
TT14	*metC1975*::Tn*10*	Anderson & Roth (1978)
TT126	*tyrA555*::Tn*10*	Anderson & Roth (1978)
TT142	*argG1828*::Tn*10*	Anderson & Roth (1978)
TT146	*argA1832*::Tn*10*	Anderson & Roth (1978)
TT169	*serA977*::Tn*10*	Anderson & Roth (1978)
TT173	*cysC1511*::Tn*10*	Anderson & Roth (1978)
TT215	*lysA565*::Tn*10*	Anderson & Roth (1978)
TT278	*guaA554*::Tn*10*	Anderson & Roth (1978)
TT287	*purC882*::Tn*10*	Anderson & Roth (1978)
TT315	*purG1739*::Tn*10*	Anderson & Roth (1978)
TT317	*purF1741*::Tn*10*	Anderson & Roth (1978)
TT418	*glyA540*::Tn*10*	Anderson & Roth (1978)
TT11	*purI1757*::Tn*10*	This paper
TT28	*hisC8579*::Tn*10*	This paper
TT98	*trp-1013*::Tn*10*	This paper
TT464	*pyrF696*::Tn*10*	This paper
TT520	*srl-202*::Tn*10*	This paper
TT1339	*pheA554*::Tn*10*	This paper
TT1454	*aroD553*::Tn*10*	This paper
TT1518	*tre-57*::Tn*10*	This paper
NK186	*cysA1539*::Tn*10*	N. Kleckner
TT2106	*hisH9528*::Tn*5*	This paper
TT2420	*hisO2355 srl-201 lysA565*::Tn*10*	TT215 × TR4179

All strains are derivatives of *S. typhimurium* strain LT2. See Materials and Methods for the derivation of strains original to this paper.

(*metA22 recA1 strA* HFr*B2*) with recipient strain TR4179. Streptomycin-resistant (Met$^+$) clones were selected and the desired recombinant was identified among the progeny. Strains TR5368 (*hisO2355*) and TR5369 (*hisO2355 recA1*) were obtained as Srl$^+$ transductants from a cross between donor strain TR2951 (*hisΔ63 recA1 strA*) and recipient strain TR4179 (*hisO2355 srl-201*). They are therefore isogenic, except for *recA*.

Strain TT2279 [*hisΔ515(gnd*$^-$*rfb*$^-$*) eda-100 met-521*/F′$_{400}$*hisO2355 gnd*$^+$*rfb*$^+$ *zzf-20*::Tn*10*] was constructed in a series of steps from strain TR3629 [*hisΔ515(gnd*$^-$*rfb*$^-$*) met-521*]. An *eda*$^-$ mutation was introduced into strain TR3629 according to the general procedures of Peyru & Frankel (1968). The deletion mutation *hisΔ515* harbored by TR3629 extends well beyond the *his* region, removing also the genes *gnd* (encoding gluconate-6-phosphate

dehydrogenase) and *rfb*. Diethylsulfate mutagenesis of strain TR3629 followed by 2 cycles of penicillin enrichment for Glk$^-$ (gluconate non-utilizing) clones yielded strain TR3949 [*his*Δ*515* (*gnd*$^-$*rfb*$^-$) *met-521 eda-100*]. It was further characterized as being unable to utilize either D-gluconic or D-glucuronic acid as a sole carbon source. Both mutagenesis and penicillin enrichment were performed according to the procedures of Roth (1970). Strain TR3951 was then constructed by mating recipient strain TR3949 with donor strain TR4520 (*his*Δ*515 arg-1819 recA1*/F$'_{400}$ *his*Δ*2634*), selecting Glk$^+$. F$'_{400}$ carries the *Salmonella* genes for *his*, *gnd* and *rfb* (Voll, 1972). To prevent recombination between this *Salmonella* episome and the chromosome, it is necessary that the chromosome either be *recA*$^-$ or harbor deletion mutation *his*Δ*515*. This deletion is very large and apparently removes all chromosomal sequences carried by F$'_{400}$. F$'_{400}$ is therefore stably maintained over the chromosome of TR3949, and the resulting merogenote (TR3951) is phenotypically His$^-$Glk$^+$Rfb$^+$. Strain TR3951 was then used as a transductional recipient for donor strain TR3578 (*hisO2355*), selecting His$^+$. The large size of deletion *his*Δ*515* prevents chromosomal His$^+$ recombinants; one episomal recombinant is strain TR4190 [*his*Δ*515* (*gnd*$^-$*rfb*$^-$) *eda-100 met-521*/F$'_{400}$*hisO2355 gnd*$^+$*rfb*$^+$]. Finally, recipient strain TR4190 was transduced with donor strain TT624 (*proAB47 arg1539 recA1 strA*/F$'$*pro*$^+$*lac*$^+$ *zzf-20*::Tn*10*), selecting tetracycline resistance. Donor strain TT624 harbors an insertion of the Tn*10* element (isolated by F. Chumley) into F-sequences of an *E. coli* F$'$*pro*$^+$*lac*$^+$ episome. This Tn*10* insertion may be transduced onto any F$'$ element, thus providing a selectable marker for subsequent F$'$ transfer (tetracycline resistance). One such Tet^R recombinant is strain TT2279 [*his*Δ*515* (*gnd*$^-$*rfb*$^-$) *eda-100 met-521*/F$'_{400}$*hisO2355 gnd*$^+$*rfb*$^+$*zzf-20*::Tn*10*].

A large number of auxotrophic and fermentation-defective mutants resulting from insertion of the Tn*10* element have been isolated in our laboratory as a co-operative effort. The sites of insertion have been identified in many of these mutants by as many as 3 independent tests: (i) the ability of selected biosynthetic intermediates to fulfill nutritional requirements (crystal tests); (ii) a demonstration of transductional linkage of Tn*10* insertions to known genetic markers; and (iii) complementation tests between Tn*10* insertions and F$'$ episomes of known genotype. The results of these tests have led to the unambiguous assignment of many Tn*10* insertions to defined genes. Tn*10* insertions into 58 such genes have thus far been identified. A small number of auxotrophs resulting from insertion of the translocatable kanamycin-resistance determinant Tn*5* (Berg *et al.*, 1975) have similarly been isolated and identified. Insertion mutations of interest to this study are shown in Table 1. Strains TT1984 to 1991 were constructed by transducing *his*$^-$::Tn*10* insertions into strain TR4179, selecting tetracycline resistance. The desired recombinants (*hisO2355 his*$^-$::Tn*10 srl-201*) were identified among the progeny as being unable to donate *hisO*$^+$ by transduction.

(c) *Genetic techniques*

(i) *Isolation of amino-triazole-resistant mutants*

In order to measure the frequency of AT†-resistant mutants, nutrient broth-grown cultures of TR5368 (*hisO2355*) or TR5369 (*hisO2355 recA1*) were diluted, and samples were spread onto minimal medium containing adenine, thiamin, and 1·8 mM-amino-triazole. Following 2 days incubation, the number of AT-resistant clones was counted.

For the isolation and analysis of independent AT-resistant mutants, individual nutrient broth cultures were inoculated with single colonies of either strain TR4179 (*hisO2355 srl-201*) or TR4255 (*hisO2355 srl-201 recA1 strA*). Following overnight growth, samples were spread individually onto minimal medium containing adenine, thiamin, and 1·8 mM-amino-triazole. From each culture, one AT-resistant mutant was picked, purified, and further analyzed.

(ii) *Duplication mapping*

The mapping data presented in Tables 3 and 4 was obtained by a spot-test method. A P22 transducing lysate grown on a particular Tn*10* auxotroph was spread on each of 2 minimal plates containing tetracycline (10 μg/ml). One of the plates also contained the

† See footnote to p. 53.

TABLE 2

hisD *enzyme levels in haploid and amino-triazole-resistant strains*

Strain	Genotype	Phenotype	Ploidy	HDHase specific activity‡
LT2	wild-type	AT^R	haploid	16·30
TR4179	*hisO2355*	AT^S	haploid	0·40
TR5401	*hisO2355/hisO2355*	AT^R	diploid	0·67
TR5402	*hisO2355/hisO2355*	AT^R	diploid	0·67
TR5536	*hisO2355*	AT^S segregant of TR5401	haploid	0·42
TR5537	*hisO2355*	AT^S segregant of TR5402	haploid	0·46

Histidinol dehydrogenase was assayed by the procedures of Ciesla *et al.* (1975). Cells were grown in minimal medium plus 0·1 mM-histidine. A *hisD* deletion mutant shows $<0·01$ unit of activity in this assay. Figures are averages of 3 or more determinations.

† AT^S and AT^R indicate sensitivity and resistance to media containing 1·8 mM-3-amino-1,2,4-triazole, respectively.

‡ Histidinol dehydrogenase (HDHase) specific activity is expressed as (cts/min conversion of [^{14}C]histidinol to [^{14}C]histidine/30 min per O.D.$_{650}$ cells) $\times 10^{-4}$.

nutritional requirements of the donor Tn*10* auxotroph. Strains carrying duplications to be mapped were spotted onto these plates (10^8 cells of each of 20 strains). Under these conditions, selection is made for Tet^R with or without a concomitant selection for prototrophy. If a given recipient yields approximately equal numbers of transductants on the 2 media ($\pm 10\%$) the duplication is judged to include the site of the Tn*10* insertion. If the minimal plate yielded few recombinants (1 to 5% of the number seen on the supplemented plates) then it was concluded that the site of the Tn*10* insertion lies outside the duplicated segment. In most cases if Tn*10* was thought to be within a duplication, several prototrophic, Tet^R transductants were purified and shown to yield segregants of the 2 parental types (Tet^R, auxotrophic or Tet^S, prototrophic).

(iii) *Other genetic techniques*

The techniques of transduction, F′ transfer, and preservation of unstable strains have been previously described (Roth, 1970; Anderson & Roth, 1978). A non-lysogenizing derivative of the high-transducing phage of Schmieger (1971), P22 HT105/1 *int-201*, was used in all transductions.

(d) *Biochemical techniques*

Radiometric assay of histidinol dehydrogenase (EC 1.1.1.23) was performed according to the procedures of Ciesla *et al.* (1975).

3. Results

(a) *Isolation of strains having tandem duplications*

We sought to develop conditions which inhibit the growth of haploid strains but allow the growth of strains merodiploid for the *his* operon. These conditions permit direct selection of strains carrying tandem duplications of the histidine operon since the gene dosage increase afforded by an extra copy of the *his* operon is essential for growth. We have developed such conditions by challenging *hisO* "promoter-like" mutants with the histidine analogue 3-amino-1,2,4-triazole, an inhibitor of histidine biosynthesis. Promoter-like *hisO* mutations are a class of *cis*-dominant regulatory mutations located at one end of the operon; they cause reduced (but regulated) levels

TABLE 3

Merodiploidy harbored by independent amino-triazole-resistant mutants

	Marker tested																		
	pyrF	*trp*	*aroD*	*tre*	*his*	*purF*	*cysA*	*purC*	*purI*	*guaA*	*glyA*	*purG*	*tyrA*	*pheA*	*cysC*	*argA*	*lysA*	*serA*	*metC*	*argG*
Map position (min):	34	34	36	37	44	49	52	54	54	55	57	57	59	59	60	61	62	63	66	68
AT-resistant mutant class																				
Class I (42 isolates)	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−
Class II (1 isolate)	−	−	−	−	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−
Class III (34 isolates)	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−

Merodiploidy was tested as described in the text. Markers are listed along the top in their relative order upon the linkage map of *S. typhimurium*. A "+" indicates that each AT-resistant mutant of that particular class was tested and found to be merodiploid for the marker shown: a "−" indicates that each AT-resistant mutant was tested and found to be haploid. Donor Tn*10* insertion strains used for testing merodiploidy were TT11, TT14, TT28, TT98, TT126, TT142, TT146, TT169, TT173, TT215, TT278, TT287, TT315, TT317, TT418, TT464, TT1339, TT1454, TT1518 and NK186. See Table 1 for their genotypes. All crosses were performed as described in Materials and Methods

TABLE 4

Amino-triazole-resistant mutants are merodiploid for all his *genes*

	Phenotype of Tet^R transductants obtained using indicated donor Tn*10* insertion							
	hisG$^-$::Tn*10*	*hisD*$^-$::Tn*10*	*hisC*$^-$::Tn*10*	*hisB*$^-$::Tn*10*	*hisH*$^-$::Tn*10*	*hisA*$^-$::Tn*10*	*hisF*$^-$::Tn*10*	*hisI*$^-$::Tn*10*
Recipient strain								
LT2	His$^-$	His$^-$	His$^-$	His$^-$	His$^-$	His$^-$	His$^-$	His$^-$
hisO2355	His$^-$	His$^-$	His$^-$	His$^-$	His$^-$	His$^-$	His$^-$	His$^-$
TR5401 (AT^R mutant)	His$^+$AT^S	His$^+$AT^S	His$^+$AT^S	His$^+$AT^S	His$^+$AT^R	His$^+$AT^R	His$^+$AT^R	His$^+$AT^R

Tetracycline-resistant recombinants were selected on nutrient broth plates containing tetracycline. Recipient strain TR5401 was obtained as a spontaneous AT-resistant mutant of *hisO2355* and was pre-grown in selective media (1·8 mM-AT) in order to decrease the number of haploid segregants in the population. Transductants were picked and subsequently tested for their His and AT phenotypes. Donor Tn*10* insertion strains used (TT1984 to TT1991); all were shown to carry the *hisO2355* mutation. See Table 1 for donor genotypes. All crosses were performed as described in Materials and Methods.

TABLE 5

Cotransduction of class III amino-triazole-resistance with lysA

	Recombinants in cross with recipient TT2420 (*hisO2355 lysA565*:Tn*10*)		
	No. Lys^+AT^S	No. Lys^+ AT^R	% Cotransduction
TR5435 (class III AT^R)	823	17	2·0
TR5464 (class I AT^R)	1304	0	<0·08
LT2 (wild-type)	420	0	<0·3

Lys^+ recombinants were selected on minimal medium plus histidine plates. Transductants were picked and subsequently tested for their AT phenotype. All crosses were performed as described in Materials and Methods.

of *his* enzyme expression (Ely *et al.*, 1974). They have the properties of classical "promoter" mutations (Jacob *et al.*, 1964; Scaife & Beckwith, 1966). Most strains containing promoter-like *hisO* mutations exhibit sufficient *his* enzyme expression to be phenotypically His^+. However, because of their limited ability to express the operon, such mutants are quite sensitive to growth inhibition by the histidine analogue 3-amino-1,2,4-triazole. AT is a specific inhibitor of the imidazole glycerol phosphate dehydratase activity of the *hisB* bifunctional enzyme (Hilton *et al.*, 1965). Wild-type strains are resistant to the growth-inhibitory properties of this compound because of their ability to derepress *hisB* expression. Promoter-like *hisO* mutants are unable to derepress fully and therefore are sensitive to AT inhibition. At appropriate concentrations of AT, *hisO* promoter mutants give rise to unstable AT-resistant clones that prove to contain tandem duplications of chromosomal segments that include the *his* operon. The appropriate concentration of AT is the minimum necessary to inhibit growth of the *hisO* mutants. Under these conditions, the doubling of *hisB* activity achieved by the gene dosage effect of a tandem duplication is apparently sufficient to allow escape from AT inhibition. The minimum inhibitory AT concentration is different for each *hisO* mutant tested. Such differences probably reflect the maximum enzyme expression possible for various *hisO* mutants.

Growth of mutant *hisO2355* (Voll, 1967) is inhibited by concentrations of AT equal to or in excess of 1·8 mM. By comparison, growth of wild-type *Salmonella* is resistant to inhibition by AT concentrations above 40 mM. When plated on media containing 1·8 mM-AT, strain *hisO2355* gives rise to AT-resistant clones that are genetically unstable; that is, when grown non-selectively (in the presence of excess histidine), cultures of such mutants accumulate AT-sensitive progeny at high frequency (10 to 40% AT-sensitive segregants following 15 to 20 generations of non-selective growth). These AT-sensitive clones are identical in all respects to the parental mutant *hisO2355*.

The instability of AT-resistant clones is demonstrated in Figure 1. A single colony of an AT-resistant mutant obtained from *hisO2355* was inoculated into nutrient broth and grown for 10 to 15 generations. This culture was diluted and samples were spread onto plates containing 1·8 mM-AT. Following three days growth, two colony types are evident: large, AT-resistant and tiny, AT-sensitive clones. The tiny colonies are characteristic of the parental mutant *hisO2355*. Such clones are stably AT-sensitive, giving rise to AT-resistant mutants only at the frequency characteristic of

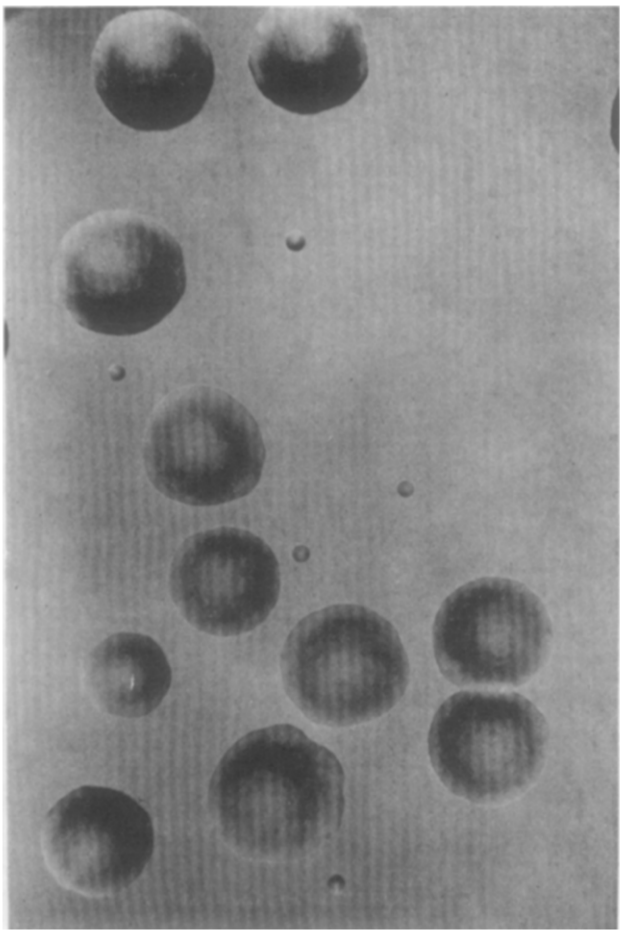

FIG. 1. Colony morphology of AT-resistant mutants and their AT-sensitive segregants.

hisO2355 (see section (g), below). The small size of these clones demonstrates the sensitivity of haploid *hisO2355* strains to AT inhibition. The large colonies in Figure 1 are those of the AT-resistant mutant. Despite repeated isolation and purification, these clones continue to segregate AT-sensitive progeny at high frequency. Thus, they are genetically unstable for their selected phenotype.

Instability is a standard feature of tandem genetic duplications (Campbell, 1963). The results presented below (sections (b) to (f)) indicate that these AT-resistant mutants harbor tandem duplications of chromosomal segments that include the *his* operon. In later sections ((g) to (h)), the formation mechanisms and adaptive significance of tandem duplication are considered.

(b) *Instability is dependent upon recombination*

Tandem genetic duplications generate haploid segregants as the result of homologous recombination between duplicated regions (Campbell, 1963). Tandem duplications are, therefore, stable in recombination-deficient backgrounds. The crosses shown in

Figure 2 demonstrate that the instability of AT-resistant mutants is dependent upon recombination (*recA* function). A *recA*$^-$ mutation was introduced into AT-resistant mutants by use of the transductionally linked marker *srl*$^-$. A *srl*$^-$ strain is unable to utilize D-sorbitol as a sole carbon source. As diagrammed in the first cross shown in Figure 2, a *srl*$^+$ *recA*$^-$ donor was used to transduce a number of independent *srl*$^-$ *recA*$^+$ AT-resistant mutants, and the isogenic Rec$^+$ and Rec$^-$ recombinants were

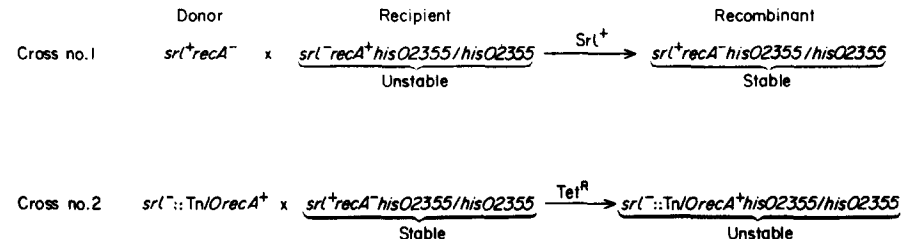

FIG. 2. Transductional crosses demonstrating that instability of AT-resistant mutants is dependent upon recombination. Selected phenotypes are indicated above the arrows. Donor strains used were TR2951 (*hisΔ63 srl*$^+$ *recA1 strA*) and TT520 (*srl-202*::Tn*10 recA*$^+$).

identified among the Srl$^+$ progeny by their ultraviolet sensitivity. Rec$^+$ recombinants (10 were tested) exhibit the same high frequency of AT-sensitive segregants characteristic of AT-resistant mutants (average = 33% following 15 to 20 generations on non-selective growth for the 4 strains tested). Rec$^-$ recombinants (10 were tested) exhibit no segregation of AT-sensitive clones (<0·008%). The second cross shown in Figure 2 demonstrates that these stable strains still harbor the duplication. Donor strain TT520 (*srl-202*::Tn*10*) was used to transduce the Rec$^-$ stable recombinants obtained from cross number one, selecting tetracycline resistance. Strain TT520 harbors an insertion of the translocatable tetracycline resistance determinant Tn*10* (Kleckner *et al.*, 1975) into the *srl* gene. Many transducing fragments that carry the *srl*$^-$::Tn*10* region of the strain TT520 also carry *recA*$^+$. Expression of such transducing fragments in infected cells yields *recA*$^+$ function (at least transiently). Homologous recombination then proceeds to integrate *recA*$^+$-transducing fragment DNA into the recipient chromosome. In this manner, *recA*$^+$ alleles were re-introduced into the Rec$^-$ stable duplications; *recA*$^+$ and *recA*$^-$ recombinants were identified by their u.v.-sensitivity. When this was done, the instability characteristic of AT-resistant mutants reappeared in all of the six *recA*$^+$ recombinants tested; none of six *recA*$^-$ recombinants tested regained instability. From these experiments, we conclude that AT-resistant mutants are at least 4000-fold more stable in *recA*$^-$ backgrounds. Thus, the genetic instability of AT-resistant mutants is dependent upon recombination.

(c) *Amino-triazole-resistant mutants have approximately twofold increased* his *enzyme levels*

If AT-resistant mutants harbor tandem duplications, their merodiploidy should be reflected by increased levels of *his* biosynthetic enzymes. A number of AT-resistant mutants have been assayed for their content of histidinol dehydrogenase, the product of the *hisD* gene. Expression of *hisD* is under the exclusive control of the primary *his* promoter (Ely & Ciesla, 1974). The level of this enzyme should reflect the gene dosage of *hisD*. Representative results of these measurements are presented in Table 2.

Two independent AT-resistant mutants obtained from *hisO2355* exhibit nearly twofold (1·7×) increased expression of *hisD* enzyme (lines 2, 3 and 4). The increase is less than twofold, probably because of haploid segregants among the diploid population at the time of assay. (Assays under repressed conditions require the addition of histidine to the growth media. Thus, haploid segregants are not counter-selected.) AT-sensitive segregants arising from AT-resistant mutants exhibit enzyme levels characteristic of haploid *hisO2355* (lines 5 and 6). The *hisD* enzyme levels exhibited by 1·8 mM-AT-resistant mutants have never been observed to be greater than twice the level of *hisO2355*. We therefore feel that AT-resistant mutants isolated in this manner contain only two copies of the *his* operon.

(d) hisO2355 *merodiploids constructed by standard genetic procedures are amino-triazole-resistant*

If AT-resistance can be caused by a tandem genetic duplication, then *hisO2355* merodiploids constructed by more standard genetic procedures should also be AT-resistant. We have constructed such a merodiploid by mating donor strain TT2279 [*hisΔ515*(*gnd*$^-$ *rfb*$^-$) *eda-100 met-521*/F$'_{400}$ *hisO2355 gnd*$^+$*rfb*$^+$ *zzf-20*::Tn*10*] (see Materials and Methods for its construction) with recipient strain TR5369 (*hisO2355 recA1*), selecting tetracycline-resistant conjugants. Donor strain TT2279 harbors an F'*his* episome of *Salmonella* origin, into which the mutation *hisO2355* and an insertion of the translocatable tetracycline-resistance determinant Tn*10* (*zzf-20*::Tn*10*) have been transduced. The Tn*10* insertion present in this strain provides a selectable marker for episome transfer (tetracycline resistance) that is unrelated to the *his* operon. Exconjugants that arise from this mating (*hisO2355 recA1*/F$'_{400}$ *hisO2355 zzf-20*::Tn*10*) contain two copies of the *hisO2355* operon. One such exconjugant (TT2280) was tested and found to be AT-resistant. We conclude, therefore, that the approximately twofold increase in gene dosage resulting from the presence of the F′ episome is sufficient to confer AT-resistance to strains harboring mutation *hisO2355*.

(e) *Amino-triazole-resistant mutants are merodiploid for large chromosomal regions*

The presence of duplicated DNA in AT-resistant mutants has been demonstrated genetically. The basic scheme for this has been to determine whether AT-resistant mutants can be made heterozygous for genetic markers near the histidine operon. If they can, then these strains must be merodiploid for those markers. To do this, we have isolated and characterized a large collection of auxotrophic and fermentation-defective mutants generated by insertion of the transposable tetracycline-resistance determinant Tn*10*. These mutations result from the linear insertion of the Tn*10* element into defined structural genes (Kleckner *et al.*, 1975).

Such mutations may be transduced selectively into recipient strains, because the Tn*10* element itself specifies a selectable phenotype (tetracycline resistance) as well as causing the mutant phenotype. Selection for inheritance of Tn*10* (by selecting tetracycline resistance) demands that recipient cells inherit the lesion caused by Tn*10* insertion. Thus, when haploid strains inherit such Tn*10* insertions, they also inherit the donor's auxotrophy or fermentation defect. If, however, the recipient strain is merodiploid for the Tn*10* insertion site, tetracycline-resistant recombinants remain prototrophic. This is due to the presence of a second (wild-type) copy of the gene

involved and the recessive nature of these mutations. Transductional crosses of this type allow rapid testing of whether a given strain is merodiploid for known Tn*10* insertion sites.

The merodiploidy harbored by 77 independent AT-resistant mutants has been determined using this technique. Our method of isolating AT-resistant mutants guarantees them to be of independent origin (see Materials and Methods). The results of these tests are presented in Table 3. Each of 77 independent AT-resistant mutants is merodiploid for a contiguous array of chromosomal loci that includes the *his* operon. One minor and two major classes of duplications are revealed. Class I AT-resistant mutants (42 independent isolates) are merodiploid for the nearby loci *purF* and *cysA*. Isolates of this type are merodiploid for 8 to 17% of the *Salmonella* genome. Class III AT-resistant mutants are merodiploid for each of 13 loci tested extending from *his* through *lysA*. Such strains are merodiploid for 18 to 26% of the chromosome. A single AT-resistant mutant (class II) is merodiploid for each of 10 loci extending from *his* through *pheA*.

None of the 77 AT-resistant mutants tested above was found to be merodiploid for the closest testable marker counter-clockwise from the *his* operon (*tre*$^-$::Tn*10*). This raised the possibility that not all *his* genes are included in the duplicated material. Since the selection for AT resistance was thought to depend upon a gene dosage increase of *hisB* (the fourth structural gene distal to *hisO*), the duplications might have been structured such that other *his* genes were not included in the duplicated material. This possibility has been eliminated by the following experiment.

Among our collection of *his*$^-$::Tn*10* insertion mutants are those in which the sites of Tn*10* insertion (a strongly polar transcriptional block) within the *his* operon have been mapped (M. Johnston, unpublished data). Tn*10* insertions have been identified in every *his* gene except *hisE*. Using the same rationale described above for testing chromosomal merodiploidy, these Tn*10* insertions may be used for testing the merodiploidy of individual *his* genes. In order to gain the most information from these crosses, it was necessary to first transduce the *his*$^-$::Tn*10* insertions into the *hisO* promoter-like mutant background. When these *hisO2355 his*$^-$::Tn*10* strains are used as donors in crosses with AT-resistant recipients (selecting tetracycline resistance), the phenotypes of the recombinants yield information which confirms that (i) AT-resistant mutants are merodiploid for all *his* genes and (ii) a doubling of *hisB* expression is the basis for AT-resistance. These conclusions are based upon the data presented in Table 4. Eight different *hisO2355 his*$^-$::Tn*10* donors were used to transduce haploid and AT-resistant merodiploid recipients, selecting tetracycline resistance. The phenotypes of the resulting recombinants are presented in Table 4. Whereas haploid recipients inherit the donor's histidine auxotrophy, AT-resistant recipients do not. Thus, AT-resistant mutants are merodiploid for all *his* genes tested (*hisG-hisI*). Furthermore, when the donor's Tn*10* insertion site is within or operator-proximal to *hisB*, tetracycline-resistant recombinants are rendered AT-sensitive. (Strains with operator-proximal insertions are actually slightly more resistant to AT than haploid *hisO2355*, due to the presence of a low-level constitutive promoter located between the *hisC* and *hisB* genes (Atkins & Loper, 1970); they are not however, fully AT-resistant.) Insertions distal to *hisB* yield fully AT-resistant His$^+$ recombinants. Considering the strongly polar nature of Tn*10* insertions (Kleckner *et al.*, 1975), these results confirm that the basis for duplication selection is an increase in *hisB* expression. The results also demonstrate that duplications include the entire *his* operon.

(f) *The large merodiploid state of amino-triazole-resistant mutants is transducible*

In strains harboring tandem genetic duplications, sequences that are widely separated in a normal chromosome are made contiguous at the junction between duplicated segments. Transduction of this novel sequence (often termed the "novel-joint"; Hershey, 1970) into normal (haploid) recipients promotes re-establishment of the donor's tandem duplication state in recipient cells. This is possible even when the amount of material included in the duplication is much too large to be carried by a single transducing fragment. Recombination events that account for this behavior are shown in Figure 3. A transducing fragment that carries the join-point of a tandem

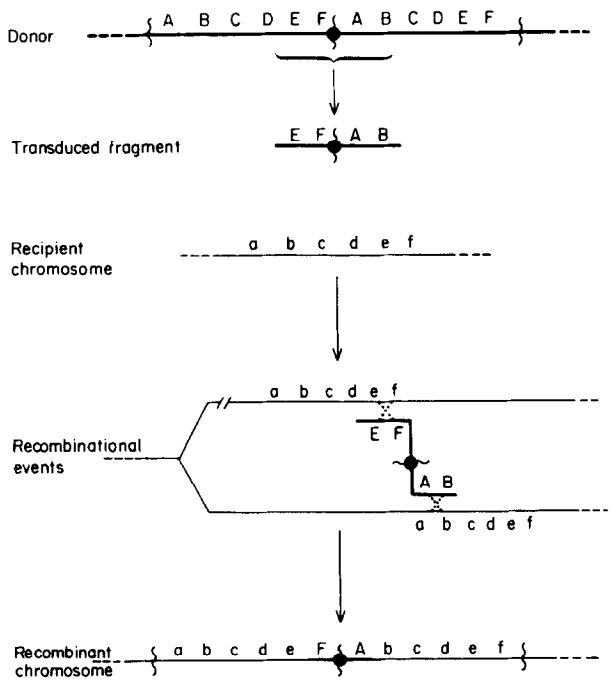

FIG. 3. A mechanism for transduction of large tandem duplications (Campbell, 1963). Recipient and donor DNAs are light and bold lined, respectively. Solid lines represent double-stranded DNA. Broken lines represent reciprocal recombination events.

duplication contains base-sequence homology to two widely separated regions of the recipient chromosome. When such a fragment enters a recipient cell, reciprocal recombination events between that fragment and *two* recipient chromosomes serve to regenerate the duplication state of the donor. In the resulting recombinant, most of the duplicated material is derived from recipient chromosomes; only material immediately adjacent to the join-point is derived from the donor. Thus, transduction of the merodiploid state of large tandem duplications may be detected, provided that a selectable donor marker and the join-point between duplicated donor segments are cotransducible. Transduction events such as those illustrated in Figure 3 were first suggested by Campbell (1965). Strong genetic evidence in support of these events has been subsequently presented (Hill *et al.*, 1969; Anderson *et al.*, 1976; Anderson & Roth, 1978).

For AT-resistant mutants of the class III type, the chromosomal location of the

duplication join-point and the ability of this join-point to promote merodiploid transductant formation may be demonstrated quite dramatically. Class III mutants are merodiploid for all loci tested in the region from *his* through *lysA* (see Table 3). They are not merodiploid for *serA* or more distal genes. *lysA* and *serA* are separated by less than one minute on the linkage map of *Salmonella* (Sanderson & Hartman, 1978). The duplication join-point in class III AT-resistant mutants may be shown to be linked to the *lysA* gene by the following experiment: when a class III AT-resistant mutant is used as a transductional donor and a *lysA*$^-$ *hisO2355* haploid strain is used as a recipient, a low but consistent fraction ($\sim$2%) of Lys$^+$ recombinants inherit the AT-resistant phenotype *non-selectively*. These data are presented in Table 5. The resulting Lys$^+$ AT-resistant recombinants are unstable for both their Lys$^+$ and AT-resistance phenotypes. They have thus inherited the large chromosomal merodiploidy characteristic of class III AT-resistant mutants. We presume that these transductants arise by events outlined generally in Figure 3 and specifically in Figure 4. We interpret these results as indicating cotransduction between the *lysA* gene and the join-point of a tandem chromosomal duplication harbored by class III AT-resistant mutants. Linkage data of this type are strong evidence that a tandem chromosomal duplication is contained by AT-resistant mutants.

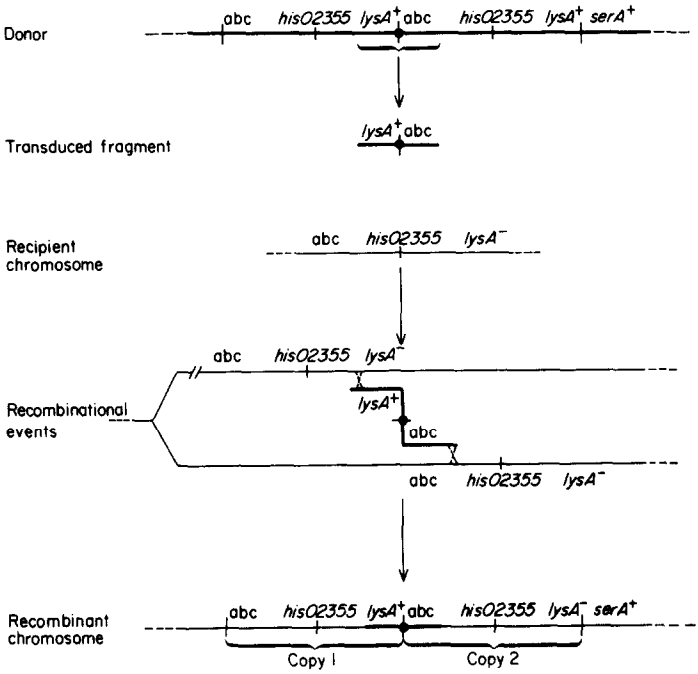

FIG. 4. Events involved in transducing the large merodiploidy harbored by class III AT-resistant mutants. Recipient and donor DNAs are light and bold-lined, respectively. Lower case letters abc are non-genetic indications of hypothetical base sequences.

(g) *Frequency of tandem duplications*

Tandem duplications of the *his* operon are frequent mutational events in *Salmonella*. Spontaneous AT-resistant mutants of strain TR5368 (*hisO2355*) occur at a frequency

of $(6{\cdot}2 \pm 3{\cdot}1) \times 10^{-5}$ per cell (average ±S.D. of 4 determinations). Of 238 AT-resistant mutants that have been purified and tested for stability, 232 were found to be unstable and are therefore tandem duplications. The mechanism of formation of these duplications is highly dependent upon recombination. Spontaneous AT-resistant mutants of strain TR5369 (*hisO2355 recA1*) occur at a frequency of $(7{\cdot}3 \pm 4{\cdot}0) \times 10^{-7}$ per cell (average ±S.D. of 4 determinations). This value is 85-fold less than the frequency for the isogenic (see Materials and Methods) *recA*$^+$ strain. Moreover, none of 76 independent AT-resistant mutants of *rec*$^-$ strain TR4255 (*hisO2355 srl-201 recA1 strA*) harbors a tandem duplication of the *his* operon. When a *recA*$^+$ allele is transduced into these 76 AT-resistant mutants, none becomes unstable. The *recA*$^+$ derivatives of all 76 strains are haploid for the *his* operon, as evidenced by their His$^-$ phenotypes upon inheritance of *his*$^-$::Tn*10* insertions. Thus, tandem duplications of the *his* operon are formed in a *recA*$^-$ background at a frequency of less than 10^{-8} per cell ($1/76 \times 7{\cdot}3 \times 10^{-7}$). This is a 6000-fold reduction of the frequency of tandem duplications seen in *recA*$^+$ cells.

(h) *Amplification of the duplicated segment*

Tandem chromosomal duplications are inherently unstable structures. Normal recombination between the two copies of duplicated material lead either to loss of the duplication (Campbell, 1963; see section (a), above) or to triplication of the segment (Sturtevant, 1925; Parma *et al.*, 1972). Cells harboring tandem triplications may be detected among cultures of AT-resistant duplications by virtue of an increased resistance to AT. When AT-resistant duplications are cultured under selective conditions (in media containing 1·8 mM-AT) for 20 to 40 generations, and samples are spread onto AT-containing plates, approximately 1 to 5% of the colonies are larger and more granular in appearance than those of duplication-containing strains. When purified and retested, the growth of these larger clones is consistently more resistant to AT inhibition than is growth of duplication-containing strains. For the following reasons such clones are thought to harbor a tandem triplication of the chromosomal segment that is duplicated in their AT-resistant parents: (i) two types of segregants arise from these clones. One type is AT-sensitive (haploid). Another type has the colony morphology, AT-resistance levels, and instability characteristic of duplication-containing strains. (ii) Transduction of auxotrophic Tn*10* insertions into these strains (see section (e), above) indicates that the chromosomal region carried in diploid (or greater) amount by these strains is the same as that carried by the parental duplication. (iii) Triploidy has been demonstrated genetically. We have in our stock collection *his*$^-$ mutations generated by insertion of the transposable *kanamycin*-resistance determinant Tn*5* (Berg *et al.*, 1975), as well as those resulting from Tn*10* insertion. One such Tn*5* insertion (isolated by D. Stetler) is within *hisH*. The more highly AT-resistant strains (triploids) were transduced with this donor (selecting kanamycin resistance), and the resulting recombinants are phenotypically His$^+$AT^{R}Kan^R (the site of Tn*5* insertion is operator-distal to *hisB*; thus recombinants remain highly AT-resistant). Such a recombinant was then used as a recipient for donor strain *hisH*$^-$::Tn*10*, selecting teracycline resistance (and kanamycin resistance). The resulting recombinants are phenotypically His$^+$AT^{R}Kan^{R}Tet^R. Moreover, when these strains are grown non-selectively three types of haploid segregants are found: (a) His$^+$AT^{S}Kan^{S}Tet^S clones (= *hisO2355*); (b) His$^-$ Kan^{R}Tet^S clones (= *hisH*::Tn*5*);

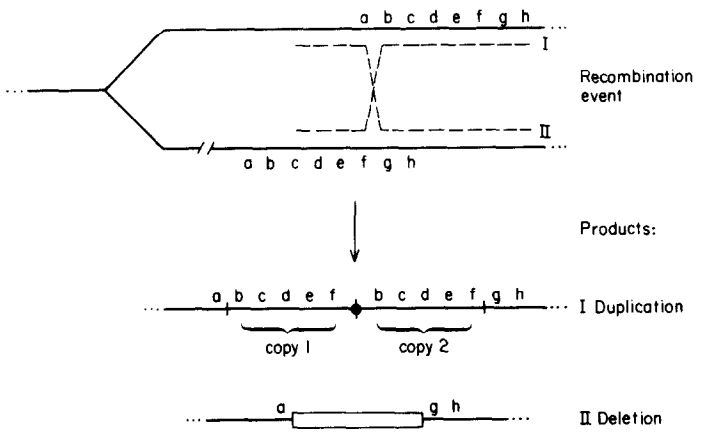

FIG. 5. Unequal crossing-over results in duplication (I) or deletion (II) of the chromosomal segment bcdef. Solid lines indicate double-stranded DNA. Broken lines represent a reciprocal recombination event.

and (c) $His^-Kan^STet^R$ clones (= *hisH*$^-$::Tn*10*). The fact that these higher-level AT-resistant strains are phenotypically His^+ even after two different *hisH* insertions have been transduced into them confirms that they are genetically triploid for the *his* operon.

The frequency of triploids among cultures of duplication-containing strains is difficult to estimate. The growth advantage of triploids when compared to diploids in AT-containing media is slight. Thus, it is not possible to select triploids directly at higher AT concentrations. They are most easily identified by prolonged cultivation of duplication-containing strains under selective conditions. Under these conditions, triploids have a growth advantage and increase their fraction of the population. They may then be identified visually by their colony size and appearance on AT-containing plates. It may be demonstrated qualitatively that the mechanism of forming triploids from diploids depends upon recombination (as expected). When the stable *recA*$^-$ AT-resistant duplications (see section (b), above) are cultivated selectively for 20 to 40 generations, no larger, more highly AT-resistant colonies are observed ($<0{\cdot}05\%$).

Although no direct proof has been obtained indicating that strains may be isolated which contain greater than triploid levels of amplification, we feel that continued selective cultivation of duplication and triplication-containing strains yields clones that harbor more tandem copies of the *his* operon. This observation is based upon the colony morphology and AT-resistance levels of strains that have been grown selectively for many generations.

4. Discussion

Strains harboring tandem duplications of chromosomal segments that include the histidine operon may be selected from populations of *hisO* promoter-like mutants. Such duplications exhibit resistance to the histidine analogue, 3-amino-1,2,4-triazole, because of a gene dosage effect upon expression of the *hisB* gene. These conclusions are based on a number of properties of AT-resistant mutants: (i) AT-resistant mutants are genetically unstable for their selected phenotype; such instability is dependent

upon a functional recombination system. (ii) AT-resistant mutants express approximately twice the level of *his* enzymes as parental strains. (iii) Genetic tests indicate that AT-resistant mutants are merodiploid for large segments of the chromosome. (iv) For certain AT-resistant mutants, a sequence that promotes AT-resistant merodiploid transductant formation (the join-point of a tandem duplication) may be cotransduced with a chromosomal marker distant to and unrelated to the *his* operon.

The spontaneous frequency of tandem duplications that include the *his* operon is quite high ($6{\cdot}2 \times 10^{-5}$ per cell). Most such duplications are quite large. Two major classes of tandem *his* duplications are repeatedly isolated. One class is merodiploid for ~13% of the genome, and another is duplicated for ~22% of the chromosome. The fact that large tandem duplications cause no loss of function suggests that there may be few limits upon the maximum size of duplicated segments. This in turn, permits even widely separated homologous sequences to be available for the unequal exchanges (see below) that lead to duplications. It is likely that the high frequency and large size of duplications reflect these considerations.

The mechanism for formation of tandem *his* duplications is highly dependent upon recombination. Tandem duplications are formed in *recA*$^-$ backgrounds at least 6000-fold less frequently than in *recA*$^+$ backgrounds. We interpret this as indicating that most duplications are formed by normal (homologous) recombination between identical (or nearly identical) sequences located at different points on the chromosome. Such unequal crossing-over is depicted in Figure 5. Reciprocal recombination between the homologous sites a-b and f-g results in recombinant chromosomes (I) carrying a duplication of the sequence bcdef and (II) carrying a deletion of the same sequence. Considering the large size of *his* duplications, the deletion products would surely be lethal.

The origin and nature of the homologous sequences at different chromosomal sites remains to be determined. It is attractive to speculate that IS-sequences (Starlinger & Saedler, 1972,1976) may be involved in the duplication process. Such sequences could provide the homology at separated chromosomal sites necessary for *rec*-dependent unequal crossing-over; they might also be involved in *rec*-independent exchanges since an IS sequence can promote illegitimate exchanges between one end of the IS and second points in the genome. Wild-type bacterial strains are thought to carry multiple copies of IS-sequences in their genomes (Saedler & Heiss, 1973). The sites of such sequences are largely unknown, but some have been identified as points of F-factor insertion into the chromosome (Sharp *et al.*, 1972; Ohtsubo *et al.*, 1974; Davidson *et al.*, 1975). It is interesting to note that both endpoints of the frequent class III duplications (one of which lies in the quite small *lysA–serA* interval) occur in regions of the *Salmonella* chromosome known to contain Hfr points of origin (Sanderson *et al.*, 1972). Ribosomal RNA genes have been shown to serve as regions of homology for duplication formation in *E. coli* (Hill *et al.*, 1977). Genes for transfer RNAs might also provide homologous sequences.

The high frequency of tandem duplications and the ability to increase the number of copies of duplicated material suggest that bacteria might utilize this process as a form of gene amplification. The adaptive role that such amplification may play in population dynamics is apparent. Any selective conditions that favor cells containing two (or more) copies of a particular gene enrich a population in those cells harboring the appropriate tandem duplication. Continued selection and competition could lead

to populations with higher levels of amplification. If growth conditions change so that a particular duplication is no longer favored, the cell population is not committed to its new genotype; the instability of duplications permits return to a predominantly haploid state. In a sense, this might be thought of as a rather crude regulatory mechanism that gains its specificity from natural selection. Although we know of no direct evidence demonstrating that such amplification is important to bacterial chromosomes its role in R-factor transitioning (Rownd & Mickel, 1971) has been clearly demonstrated (Hashimoto & Rownd, 1975). A more widespread use of this process for amplification of chromosomal genes by clonal selection would not seem surprising.

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REFERENCES

Anderson, R. P. & Roth, J. R. (1977). *Annu. Rev. Microbiol.* **31**, 473–505.
Anderson, R. P. & Roth, J. R. (1978). *J. Mol. Biol.* **119**, 147–166.
Anderson, R. P., Miller, C. G. & Roth, J. R. (1976). *J. Mol. Biol.* **105**, 201–218.
Atkins, J. F. & Loper, J. C. (1970). *Proc. Nat. Acad. Sci., U.S.A.* **65**, 925–932.
Berg, D., Davies, J., Allet, B. & Rochaix, J. P. (1975). *Proc. Nat. Acad. Sci., U.S.A.* **72**, 3628–3632.
Campbell, A. (1963). *Virology*, **20**, 344–356.
Campbell, A. (1965). *Virology*, **27**, 329–339.
Ciesla, Z., Salvatore, F., Broach, J. R., Artz, S. W. & Ames, B. N. (1975). *Anal. Biochem.* **63**, 44–55.
Davidson, N., Deonier, R. C., Hu, S. & Ohtsubo, E. (1975). *Microbiology*, **1**, 56–71.
Ely, B. & Ciesla, Z. (1974). *J. Bacteriol.* **120**, 984–986.
Ely, B., Fankhauser, D. B. & Hartman, P. E. (1974). *Genetics*, **78**, 607–631.
Hashimoto, H. & Rownd, R. H. (1975). *J. Bacteriol.* **123**, 56–68.
Hegeman, G. D. & Rosenberg, S. L. (1970). *Annu. Rev. Microbiol.* **24**, 429–462.
Hershey, A. D. (1970). *Yearb. Carnegie Instn*, **69**, 717–722.
Hill, C. W. & Combriato, G. (1973). *Mol. Gen. Genet.* **127**, 197–214.
Hill, C. W., Schiffer, D. & Berg, P. (1969). *J. Bacteriol.* **99**, 274–278.
Hill, C. W., Grafstrom, R., Harnish, B. & Hillman, B. (1977). *J. Mol. Biol.* **116**, 407–408.
Hilton, J., Kearney, P. & Ames, B. N. (1965). *Arch. Biochem. Biophys.* **112**, 544–547.
Horiuchi, T., Horiuchi, S. & Novick, A. (1963). *Genetics*, **48**, 157–169.
Jacob, F., Ullman, A. & Monod, J. (1964). *C. R. H. Acad. Sci.* **258**, 3125–3128.
Kleckner, N., Chan, R. K., Tye, B. K. & Botstein, D. (1975). *J. Mol. Biol.* **97**, 561–575.
Langridge, J. (1969). *Mol. Gen. Genet.* **105**, 74–83.
McEntee, K. (1976). *Virology*, **70**, 221–222.
Miller, C. G. & Roth, J. R. (1971). *J. Mol. Biol.* **59**, 63–75.
Novick, A. & Horiuchi, T. (1961). *Cold Spring Harbor Symp. Quant. Biol.* **26**, 234–245.
Ohno, S. (1970). *Evolution by Gene Duplication*, pp. 160, Springer-Verlag, New York.
Ohtsubo, E., Deonier, R. C., Lee, H. J. & Davidson, J. (1974). *J. Mol. Biol.* **89**, 565–584.
Parma, D. H., Ingraham, L. J. & Snyder, M. (1972). *Genetics*, **71**, 319–335.
Peyru, G. & Frankel, D. G. (1968). *J. Bacteriol.* **95**, 1273–1278.
Rigby, P. W. J., Burleigh, B. D. & Hartley, B. S. (1974). *Nature (London)*, **251**, 200–204.
Roth, J. R. (1970). *Meth. Enzymol.* **17**, 3–35.
Rownd, R. & Mickel, S. (1971). *Nature New Biol.* **234**, 40–43.
Saedler, H. & Heiss, B. (1973). *Mol. Gen. Genet.* **122**, 267–277.
Sanderson, K. E. & Hartman, P. E. (1978). *Bacteriol. Rev.* **42**, 471–519.
Sanderson, D. E., Ross, H., Ziegler, L. & Mäkelä, P. H. (1972). *Bacteriol. Rev.* **36**, 608–637.
Scaife, J. & Beckwith, J. (1966). *Cold Spring Harbor Symp. Quant. Biol.* **31**, 403–408.
Schmieger, H. (1971). *Mol. Gen. Genet.* **110**, 378–381.

Sharp, P. A., Hsu, M.-T., Ohtsubo, E. & Davidson, N. (1972). *J. Mol. Biol.* **71**, 471–497.
Starlinger, P. & Saedler, H. (1972). *Biochimie*, **54**, 177–196.
Starlinger, P. & Staedler, H. (1976). *Curr. Top. Microbiol. Immunol.* **75**, 111–123.
Straus, D. S. (1974). *Genetics*, **78**, 823–830.
Straus, D. S. & Hoffmann, G. R. (1975). *Genetics*, **80**, 227–237.
Straus, D. S. & Straus, L. D. (1976). *J. Mol. Biol.* **103**, 143–154.
Sturtevant, A. H. (1925). *Genetics*, **10**, 117–147.
Vogel, H. & Bonner, D. (1956). *J. Biol. Chem.* **218**, 97–106.
Voll, M. J. (1967). *J. Mol. Biol.* **30**, 109–124.
Voll, M. J. (1972). *J. Bacteriol.* **109**, 741–750.