TESTS FOR ALLELISM AMONG AUXOTROPHS OF SALMONELLA TYPHIMURIUM[1]

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ONE of the important contributions of classical genetics is the evidence that a gene may exist in several forms, giving rise to different although similar phenotypes. Different forms of a gene, any one of which may be present at the gene locus, are known as alleles. The evidence now available indicates that, when one is dealing with recessive mutant characters, combinations between any allele and the wild type usually produce the wild type phenotype, and combinations of alleles produce mutant phenotypes (*trans* configurations). Early efforts to observe crossing over between alleles of a gene locus were not successful; and therefore the gene was defined as the unit of physiological function as well as of crossing over. According to this definition it should be possible, by investigating the phenotype of the heterozygote resulting from a cross of two similar mutants, and the crossover relation between the two mutants, to determine whether or not they are alleles of a single gene locus. As early as 1929, however, the validity of crossover relationship as a criterion for identifying a gene locus was called in question by the results of DUBININ's (1929) studies of relations between alleles of the scute locus in Drosophila, which suggested that crossing over may occur *within* a gene locus. Since that time, evidence has been accumulating that crossing over within a gene locus—that is, between different sites of a locus—does occur; and the findings of recent work with Salmonella (DEMEREC 1956a) make it seem probable that this is true of most gene loci, for it has been detected in every case (44 so far) in which two or more alleles have been available for study. Thus, a gene can no longer be defined as the unit of crossing over; and physiological function and physical arrangement of sites within a locus are the only known bases for identification of a gene locus.

If genes were distributed at random in chromosomes, since there are several thousand different genes present in every organism, there would be very little likelihood that two genes having similar functions would be located together, and so a gene could be identified with a fair degree of certainty by study of its behavior in recombination. There is a considerable amount of evidence, however, that the distribution of genes is not random (summarized in PONTECORVO 1956), and the recent work with Salmonella (DEMEREC 1956a) has demonstrated that genes controlling related functions are often adjacent to one another. Therefore,

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to establish whether two closely linked similar mutant genes are alleles of one gene locus or represent mutations at two loci, it is essential to investigate their functions. In auxotrophs of *Salmonella typhimurium*, this can be done very effectively by means of abortive-transduction experiments. Evidence gathered in the course of work by several members of our laboratory indicates that abortive transduction is a general phenomenon among these auxotrophs, and that abortive-transduction tests can be applied successfully to differentiate between closely linked, presumably adjacent, gene loci which control related functions.

Abortive transduction

In transduction of an auxotrophic strain of bacteria by the wild type, by a nonallelic mutant strain, or by an allelic but not identical strain, part of the donor chromosome is incorporated into a chromosome of recipient cells, changing the genotype to wild type and giving rise to wild type colonies. This process will be referred to hereafter as "complete transduction." While studying complete transduction among purine-requiring mutants of *S. typhimurium*, strain LT-2, OZEKI (1956) observed the appearance of a number of very small (minute) colonies in addition to the expected large (wild type) colonies. His analysis of these minute colonies showed that each was composed of three different kinds of cells: (1) a single progenitor cell, which carried the wild type gene introduced by phage and was phenotypically prototrophic; (2) daughter cells, produced at every division of the progenitor, which did not receive the wild type gene but could undergo a limited number of divisions on medium deficient in the growth factor required by the recipient bacteria; and (3) the final progenies of such daughter cells, which like the original recipient were not able to divide on the deficient medium. The mechanism responsible for the minute colonies appeared to resemble the process, termed by STOCKER and others (1953) "abortive transduction," which is responsible for the "trail phenomenon" in certain instances of transduction of motility in Salmonella; and the same name was applied to it.

OZEKI's work indicated that minute colonies appear whenever the donor carries the wild type allele of the mutant gene present in the recipient bacteria, and do not appear when it carries a mutant allele. Thus it seems that the appearance of minute colonies reflects functional complementation between the marker genes of the recipient and the donor, and that an abortive-transduction experiment represents a *trans*-configuration test for the markers involved. Therefore, the presence or absence of minute colonies in transduction tests involving two phenotypically similar and closely linked mutants should provide information regarding the functional difference or identity of the two genes, independently of biochemical or other tests.

TECHNICAL PROCEDURES

The procedures followed in this laboratory for detection of abortive transduction between two auxotrophs are the same as those employed in ordinary transduction experiments with these Salmonella mutants. About 10^8 bacteria from a

full-grown culture of the recipient strain, infected with about 5×10^8 phage particles derived from the donor strain, and incubated 8–10 minutes at 37°C for adsorption of the phage, are spread uniformly on agar plates.

To permit detection of the minute colonies that represent abortive transduction, the medium on which recipient bacteria are plated should allow some background growth and at the same time favor growth of the minute colonies. Therefore it is desirable, and in some instances essential, to supplement minimal medium with various compounds. As a rule, studies of abortive transduction are facilitated by enrichment of the minimal medium with a small amount of broth (0.01 percent broth powder). In the case of *tryD* mutants, the size of minute colonies is markedly increased by supplementation with 0.02 to 0.05 percent of neutralized vitamin-free casein hydrolysate (YURA, in DEMEREC *et al.* 1956); and in the case of *adeE* and *adeF* mutants, the addition of casein hydrolysate (0.1 percent) is necessary for detection of the colonies (GOTS, in DEMEREC *et al.* 1957). In work with *adthA*, *adthC*, and *adthD* bacteria, detection is facilitated by supplements of adenine and pantothenate (OZEKI 1956).

After one or two days' incubation at 37°C, the plates are observed microscopically at 10 × to 20 × magnification. Under the conditions described, minute colonies are approximately 0.05 mm in diameter, although the size depends somewhat upon the strain of the recipient bacteria as well as upon the kind of medium and length of the incubation period. Two controls are needed in every experiment: (1) platings of the recipient with phage from the wild type, and (2) platings of the recipient with phage from its own strain. Only when minute colonies are clearly observed in the first case, and no such colonies are observed in the second case, can a conclusion be drawn as to whether or not the recipient strain undergoes abortive transduction by the other auxotrophic strain being tested. In such cases the presence of minute colonies on a transductional plate indicates that the two markers are nonallelic, whereas allelism is indicated by their absence. In tests for allelism, therefore, results are scored in terms of presence (plus) or absence (minus) of minute colonies. As a rule, the number of minute colonies resulting from transduction between two nonallelic mutants is about the same as the number formed when the wild type is the donor, even if the two mutant markers are closely linked and the number of large colonies (complete transductions to prototrophy) is therefore small.

The conclusion that the minute colonies appearing in experiments are due to abortive transduction has been confirmed in a number of instances, either by streaking or by respreading. As shown by OZEKI (1956), when a minute colony is picked up and streaked on a new plate, no more than one minute colony is formed on the streak.

MATERIALS AND TERMINOLOGY

Most of the auxotrophic mutants studied were derivatives of *S. typhimurium* strain LT–2 (ZINDER and LEDERBERG 1952), and a few were derived from strain LT–7. The temperate phage employed in transduction experiments was PLT–22.

The minimal medium contained 10.5 g of K_2HPO_4, 4.5 g of KH_2PO_4, 0.05 g of $MgSO_4$, 1 g of $(NH_4)_2SO_4$, 0.47 g of sodium citrate, 2 g of glucose, and 15 g of agar per 1000 ml of demineralized water.

The terms defined by DEMEREC (1956b) will be used in this paper. The section of a chromosome occupied by one gene is called the gene *locus*. A locus can be subdivided by crossing over into linearly arranged units, which are called *sites*. The different forms in which a gene may exist are called *alleles*. They may be *nonidentical*, if due to mutations at non-homologous sites of a gene locus, or *identical*, if due to mutations at homologous sites. A *single-site* mutant is one that has not, so far, been shown to be due to mutations at more than one site in the locus. A *multisite* mutant is one that fails to give recombinants in reciprocal transduction tests with a number of closely linked markers, and may therefore be said to "cover" more than one site, either within a locus or in neighboring loci.

The symbol for a mutant character utilizes a convenient abbreviation of the auxotrophic requirement involved; for example, tryptophan, *try;* adenine, *ade;* adenine-thiamine, *adth;* histidine, *his;* cystine, *cys;* proline, *pro;* methionine, *met;* serine, *ser*. Similar mutants of independent origin are distinguished by numbers assigned in the order in which they are found, for example, *try-1*, *try-2*, *try-3*. Finally, when the gene locus has been determined, a capital letter designating the locus is included in the mutant symbol, as *tryD-1*, *tryB-2*, *tryC-3*, *tryA-8*.

Generality of abortive transduction

The usefulness of the abortive-transduction technique for allelism tests depends on how generally it can be applied. Since minute colonies result from additional divisions of cells whose progenitor contained a transducing fragment carrying the wild type allele of the mutant marker of the recipient cells, it is reasonable to assume that each such progenitor cell is phenotypically prototrophic, and that the one daughter cell which does not receive the wild type allele still is able to undergo a finite number of divisions on medium which does not support the growth of recipient cells. The implication of this assumption is that minute colonies will appear whenever the auxotrophic character is recessive to the wild type, provided the medium is suitable for their detection.

During the past two years, abortive transductions have been detected in experiments with 280 auxotrophs representing 41 different gene loci, as follows: adenine, 4; adenine-thiamine, 4; tryptophan, 4; histidine, 7; cystine, 6; methionine, 4; proline, 4; serine, 2; threonine, 4; guanine, 1; and pantothenate, 1. They have not been detected in experiments with any known allele of the leucine *A* and *B* loci, or with some alleles of certain cystine and tryptophan loci. Presumably in these cases the media used were not suitable for the detection of minute colonies.

Tests for allelism

The abortive-transduction technique is now being used in our laboratory to test for functional complementation among similar mutants and thus gain evi-

dence about their allelic relationships. A brief summary of the results will be presented here.

Purine mutants: In his paper on abortive transduction, OZEKI (1956) reported the results of experiments with *adthA-1, -2, -3, -4, -10, -18; adthC-5, -14;* and *adthD-11, -12, -17* as recipients and *adthA-1, -2, -3, -4, -8, -9, -10; adthB-6; adthC-5, -14; adthD-11, -12; adeA-1; adeC-2, -4, -8;* and *adeD-10* as donors. Minute colonies showed up in every tested combination in which the recipient and the donor belonged to different loci, and failed to show up when they were members of the same locus. In these experiments the minimal medium was supplemented with adenine and pantothenate, so that minute colonies would be easily detectable. Positive evidence of linkage between *adthB* and *adthD*, and between *adthC* and *adeC-2*, has been obtained by transduction tests (DEMEREC *et al.* 1956).

As the two mutant alleles *adeC-2* and *-7* are linked with *adthC* and *guaA*, whereas the remaining alleles of this group are not, the locus originally designated *adeC* was divided into two, *adeC (-2* and *-7)* and *adeE (-4, -5, -6, -8, -9, -11,* and *-14* through *-29)* although the mutants are phenotypically indistinguishable (DEMEREC *et al.* 1956). GOTS and GREGORY (DEMEREC *et al.* 1957) were able to observe minute colonies in transduction experiments between *adeC (-2* and *-7)* and *adeE* mutants on medium supplemented with casein hydrolysate. This result confirms the grouping shown above.

Further study of the 22 mutants of this *adeE* group has revealed at least two functionally different types; some of the mutants *(adeE-6, -8, -15,* and *-19)* form minute colonies with another of the group. Thus this group probably represents two closely linked loci, *adeE* and *adeF* (GOTS and GREGORY, in DEMEREC *et al.* 1957).

It has been shown that the mutant originally described as *adeD-10* actually belongs to the *adthD* locus; it is now called *adth D-20*. Moreover, the data suggest that the site of this mutation coincides with that of the *adthD-12* mutation, although *adthD-20* requires only adenine for growth whereas *adthD-12* requires adenine plus thiamine (OZEKI, in DEMEREC *et al.* 1956). As was mentioned earlier, the former *adeD-10*—that is, *adthD-20*—produced minute colonies when used as donor even with the other mutants of the *adthD* locus, including *adthD-12*. This observation suggests that abortive transduction as a test for allelism may not always be applicable.

Histidine mutants: Very comprehensive tests for abortive transduction between histidine mutants have been carried out by P. E. HARTMAN and his coworkers (HARTMAN, in DEMEREC *et al.* 1956; and HARTMAN *et al.*, personal communication). Eighty-four closely linked histidine mutants have been divided into five functional groups on the basis of biochemical characterization of the mutant phenotypes. More than 6000 different combinations of the mutants were tested for functional complementation by the abortive-transduction method. Bacteria of two of the groups—containing 13 and 7 mutants—produced minute colonies when tested with mutants belonging to each of the other groups and no minute colonies when tested with mutants of their own groups. This finding supported

the conclusion that these two groups differ functionally from each other and represent mutations at two gene loci (*hisC* and *hisG*). The 22 mutants of a third biochemical class, however, were clearly divided by the abortive-transduction results into three functional groups, namely, *hisE* (2 mutants), *hisF* (10), and *hisA* (10). These three loci are arranged linearly, as determined by crossing over tests with complete transduction. More recently, in work carried on at Harvard University, it has been possible to distinguish between groups *A* and *F* by biochemical criteria (MOYED and MAGASANIK 1957).

In contrast to the findings regarding the five loci just described, HARTMAN and his collaborators (personal communication) observed that some mutants of the *hisB* locus (17 mutants) and *hisD* locus (25 mutants) did not conform to the pattern of behavior generally found in abortive-transduction tests between alleles of the same locus. Minute colonies appeared in almost all possible combinations of *B* and *D* mutants with mutants of each of the other six loci. Minute colonies also appeared, however, in some intralocus combinations. These aberrant positive results occurred frequently in locus *B* tests, rarely in locus *D*. The reasons for this exceptional kind of behavior are not yet clear, but the findings of HARTMAN and his colleagues suggest that complementation cannot always be used as a test of allelism. Otherwise it would be necessary, for example, to assume that the *hisB* group represents at least four gene loci. This seems unlikely, inasmuch as the *hisB* block appears to be concerned with a single enzymatic reaction carried on by imidazoleglycerol phosphate ester dehydrase. It also leaves unexplained the negative intralocus tests between *B* mutants which are nevertheless separable by recombination.

Proline mutants: About 140 mutants defective in proline synthesis are now being investigated by T. MIYAKE. Analysis of 101 of them, all of which are single-site mutants, has progressed far enough to show that they can be divided into four discrete groups on the basis of some or all of three criteria, namely, nutritional requirements, numbers of complete transductions, and presence or absence of abortive transductions. There are 35 mutants in group *A*, 46 in *B*, 16 in *C*, and 4 in *D*. The *A* and *B* loci are closely linked, whereas *C* and *D* are independent as far as transduction tests are concerned. Abortive transductions were observed in all tested combinations of markers representing different groups, but not in any tested combination of markers of the same group. Mutants of groups *A* and *B* cannot be differentiated on the basis of known nutritional requirements, since all are able to grow on either proline or glutamic-γ-semialdehyde. By studies of the frequencies of recombination between mutants of these two groups, and also by experiments with overlapping multisite mutants, it has been possible to determine the linear order of their mutational sites on a linkage map. The positions of the markers on the chromosome correspond to the *A* and *B* groupings arrived at by abortive-transduction tests.

Cystine mutants: In accordance with the results of nutritional tests and transduction experiments, a number of cystine-requiring mutants were divided into five groups. A close linkage relation was demonstrated between two of the

groups, *C* and *D;* that is to say, markers of the *cysC* and *cysD* loci are carried by one transducing fragment (CLOWES 1958). CLOWES made tests for abortive transduction with one representative mutant of each group *(cysA-1, B-14, C-7, D-23,* and *E-30).* Tests were made with all combinations, including homologous combinations. As recipients, *cysA-1, cysB-14,* and *cysE-30* gave distinct minute colonies with any other mutant as donor; and no such colonies were observed in any homologous combination. The background growth of *cysC* and *cysD* makes detection of minute colonies difficult, so that it was impossible to apply the test to *cysC-7* and *cysD-23* as recipients.

Abortive-transduction tests among certain *cysB* mutants *(-10, -14, -15, -18,* and *-45)* were also made by CLOWES (1958). A phenotypic difference was observed among these mutants; in *cysB-10* and *-18* the nutritional requirement is satisfied by either cysteine or SO_3, whereas in *-14, -15,* and *-45* it is satisfied only by cysteine. The mutational sites of the two phenotypes are distributed alternately, in the order *14——18——45——10——15.* No minute colonies were observed in any combination among these *cysB* mutants. This indicates that there is no complementary relation between the cysteine-SO_3 type and the cysteine-only type, and that all are alleles of one locus although their phenotypes are somewhat different.

HOWARTH (1958) made abortive-transduction tests with all possible combinations of *cys-1, -3, -5, -13, -20, -21, -22, -32, -69,* and *-82.* All these mutants are closely linked; and they have similar nutritional requirements, for in all of them the cystine deficiency can be compensated for by either cysteine, sulfite, or a high concentration of thiosulfate. Therefore they had been regarded as alleles of one gene locus *(cysA).* In HOWARTH's experiments, however, minute colonies appeared when *cys-22* and *cys-32* were tested with any other member of this group except the multisite mutant *cys-20,* but not when they were tested with each other. This finding suggested that *cys-22* and *cys-32* are functionally different from the other mutants of the group, and that the group actually represents two closely linked loci. Frequencies of complete transduction—that is, frequencies of recombination—between members of the group indicated that the *cys-22* and *cys-32* mutational sites are located close together, at one end of the linearly arranged sequence, and so supported the assumption that they are members of a separate locus. This gene locus has been designated *cysF.* In experiments between *cys-20* and other members of the group, neither abortive nor complete transduction was observed, although with the wild type as donor both appeared. Thus *cys-20* is a multisite mutant, covering all known sites of the *cysA* and *cysF* loci.

Pantothenate mutants: Our collection includes three mutants whose nutritional requirements can be satisfied by calcium pantothenate. Tests of the three in various combinations yielded very few instances of complete transduction; and abortive transduction did not occur between any two of the mutants, although it did occur when each was tested with the wild type as donor. Thus it appears that the three mutants are closely linked and functionally similar, and can be considered nonidentical alleles of one gene locus.

Tryptophan mutants: By means of transduction tests and analyses of biosynthetic blocks, the 56 tryptophan-requiring mutant strains so far isolated have been placed in four closely linked groups: *tryA*, *B*, *C*, and *D* (DEMEREC and Z. HARTMAN 1956). Group *D* has 12 mutants; group *C*, 9; group *B*, 27; and group *A*, 8.

As was reported by YURA (DEMEREC *et al.* 1956), members of the *tryD* group are deficient in a single enzyme, namely, tryptophan synthetase. Abortive-transduction tests were made by Yura with the recipients *tryD-1*, *-6*, *-7*, *-9*, and *-10* and the donors *tryD-1*, *-6*, *-7*, *-9*, *-10*, *tryC-3*, *tryB-4*, *tryA-8*, and wild type. Additional experiments with *tryD* mutants were made by E. L. LAHR. Minute colonies did not appear in tests between members of the *tryD* group, but they did occur in the other combinations of the experiments. All the *tryD* mutants tested thus form a homogeneous group, both in terms of their enzyme deficiency and according to the criteria of complete and abortive transduction; and they can all be considered alleles of a single gene locus.

Similar tests were made by LAHR with 15 members of the *tryB* group. Indole supports the growth of these mutants on minimal medium, but anthranilic acid does not. Seven of them *(try-4*, *-14*, *-16*, *-17*, *-26*, *-27*, and *-34)* gave rise to minute colonies in experiments with the wild type as donor, whereas the other eight *(try-2*, *-12*, *-13*, *-19*, *-23*, *-30*, *-31*, and *-32)* did not. The minute colonies were most readily observable when casein hydrolysate was added to the medium. Experiments between members of the group as donor and recipient gave irregular results—a further indication that the present *tryB* group represents at least two gene loci, in agreement with the results of YANOFSKY (1956), who showed that there are two steps in the biosynthesis of indole from anthranilic acid.

Leucine mutants: Our collection includes 105 leucine requiring mutants belonging in two unlinked groups. E. GLANVILLE has used 39 of these in extensive experiments for the detection of abortive transduction, with negative results.

DISCUSSION

The material presented here shows that the phenomenon of minute-colony formation as a result of abortive transduction, first observed in some purine-requiring mutants by OZEKI (1956), occurs generally among auxotrophs of *Salmonella typhimurium* strains LT-2 and LT-7. It appears that the formation of minute colonies in transduction experiments with these mutants reveals functional complementation between the two markers involved; because as a rule no minute colonies are formed in combinations of markers representing nonidentical alleles of a single gene locus.

A few exceptions, such as those found by HARTMAN in the *hisB* locus, seem to resemble a type of behavior observed infrequently in Neurospora (GILES *et al.* 1957), Aspergillus (PONTECORVO 1956), and phage (KAISER 1957), and suggest that noncomplementation, as detected by abortive-transduction and heterocaryon tests, is not an essential characteristic of alleles. Thus, evidence seems to be accumulating that the differences among component parts (sites) of a gene locus,

disclosed by studies of the various properties of mutant alleles (DEMEREC 1956a), may in rare instances be such that genetic changes at certain sites produce mutants which complement mutants produced by changes at certain other sites of the same locus.

In such instances, rare at the present time, complementation alone cannot serve as the criterion for differentiating between two genes. When several functionally similar mutants belonging to two complementary groups are closely linked, the distribution of their sites determines whether they belong to a single gene locus (two kinds of sites mixed in linear sequence) or two closely related loci (each of the two kinds grouped together).

With regard to complementation, it may be pointed out that a situation similar to that observed in these auxotrophs has also been found in studies of motility in Salmonella (LEDERBERG 1956; STOCKER 1956; and more recent work in STOCKER's laboratory). In all transductional combinations of different nonmotile (*Fla*$^-$) strains so far tested, not only motile clones (resulting from complete transduction) but also trails (resulting from abortive transduction, comparable to minute colonies in auxotrophs) have been observed, whereas nonmotile strains infected with phage from the same strain never produce trails. This result indicates that these mutations to nonmotility, although they all affect the same character (formation of flagella) and many of them show linkage with another mutation, H_1, controlling flagellar antigen type (see LEDERBERG and IINO 1956), complement one another. In this case, if complementation were used as a criterion for identifying gene loci, one would have to assume that there are many different *Fla* loci, linked together and forming a cluster near the H_1 locus. It seems rather unlikely that so many loci affecting the formation of flagella should be present within the length of a transducing fragment of chromosome, and that each mutation to nonmotility found so far should have occurred at a different locus. A more plausible interpretation is the assumption that one or only a few gene loci, showing functional complementation between alleles, are involved. We wish to point out, however, that the nonmotile strains of LEDERBERG's and STOCKER's experiments were derived from different sources, including *S. typhimurium*, *S. paratyphi-B*, and *S. heidelberg*. On the other hand, the auxotrophs of the present study were all derived from two strains of *S. typhimurium* (LT-2 and LT-7). The complementation observed in experiments between different nonmotile strains might be an effect of change in genetic background on the behavior of genes participating in transduction. In other words, a mutant gene of one strain when introduced into a bacterium of another strain may be stimulated to function more actively. If this should be the case, it would call for caution in the application of complementation as a test of allelism in mutants of different species, or even of unrelated strains.

SUMMARY

In transduction experiments with auxotrophs of *Salmonella typhimurium*, the occurrence of abortive transduction is an indication of functional complementa-

tion between similar mutants. Abortive transduction has been detected in experiments with 280 auxotrophs representing 41 different gene loci, which control the synthesis of various amino acids, purines, and vitamins. As a rule, complementation may serve as the criterion for identification of closely linked functionally similar genes; but HARTMAN's studies of the *hisB* locus indicate that in some instances this criterion fails, and that other factors have to be considered in determining whether two complementary groups of closely linked mutants represent a single gene locus or two closely related gene loci.

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