Role of Gene Duplications in the Adaptation of *Salmonella typhimurium* to Growth on Limiting Carbon Sources

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ABSTRACT

Duplication-containing cells are selected when growth of *Salmonella typhimurium* is limited by the availability of any one of several carbon and energy sources. Under conditions of extreme starvation, growth occurs almost exclusively in the duplication-containing fraction of the population. Cells with duplications of one large segment of the chromosome are repeatedly selected regardless of which of these carbon sources limits growth. The duplicated chromosomal segment encodes the transport systems for all of these carbon sources. This duplication is not selected during growth on a carbon source for which the permease is not included within the duplication segment. This suggests that the growth advantage conferred by the duplication may be due to increased transport of the limiting carbon source. Inclusion of the permease alone is not sufficient to explain the growth advantage of the duplications, since other common duplications that include the permease are not selected.

GENE duplications occur at a high frequency in *Salmonella typhimurium* (ANDERSON and ROTH 1981). Most sites on the bacterial chromosome are found to be duplicated at frequencies of 10^{-3} to 10^{-4}. Gene duplications are extremely unstable and revert to the haploid condition. Haploid segregants are found at frequencies of 1–30% in overnight cultures of duplication-containing strains that are grown under nonselective conditions (SCHMID 1981). The high frequency of formation and the rapid loss of duplications suggests that gene duplications may be a mechanism by which bacteria can amplify particular functions and thereby adapt to stressful conditions in nature without having to undergo irreversible changes in their genomes. A possible example of this mechanism may be the observation that *Vibrio cholerae* cells with gene amplifications are selected during growth in the rabbit intestine (MEKALANOS 1983).

A number of investigators have shown that gene duplications are a frequency class of mutants that appear following selections for increased enzyme activity (for review see ANDERSON and ROTH 1977; also see TILSTY, ALBERTINI and MILLER 1984). Most of these selections demand growth in the presence of a particular inhibitor, growth on substrates for which an enzyme has a low affinity, or growth of mutant strains that produce an abnormally low level of a particular enzymatic activity. Selections of this kind may not be common in nature. In order to assess the possible importance of duplications in adaptation of bacteria to natural conditions, we have subjected wild-type *S. typhimurium* to selections for growth on low concentrations of a carbon source. It seems likely that such selection conditions might occur frequently in nature. We have found that there is a strong selection for duplication-containing strains under such conditions.

The majority of genetic duplications in *S. typhimurium* are large and include many genes (ANDERSON and ROTH 1981). The endpoint distribution of the duplications that are selected in our experiments suggests that the growth advantage seen here is conferred by duplicating a particular set of genes. If the ability to form particular duplications is important in nature, it may represent a selective evolutionary force for determining the location of genes on the bacterial chromosome.

MATERIALS AND METHODS

Bacteria: All strains used are derivatives of *S. typhimurium* LT2. A list of strains is shown in Table 1.

Media: Minimal medium was either the E medium of VOGEL and BONNER (1956) or NCE medium (BERKOWITZ *et al.* 1968). E medium was supplemented with glucose. NCE medium was supplemented with one of the following: L-arabinose, L-malate, D-melibiose or D-sorbitol. Unless otherwise specified, the carbon source was added at a final concentration of 0.2%. The complex medium was nutrient broth (0.8%; Difco) with added sodium chloride (0.5%). Tetrazolium indicator plates were made according to the protocol described in WINKLEMAN and CLARK (1984). Auxotrophic supplements were added to minimal media at concentrations described by DAVIS, ROTH and BOTSTEIN (1980). Antibiotics were used at the following concentrations in minimal and complex media, respectively: kanamycin: 125 μg/ml and 50 μg/ml; tetracycline: 10 μg/ml and 20 μg/ml;

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TABLE 1

Strain list

Strain No.	Genotype	Source
LT2	Wild type	Laboratory collection
TT142	*argG1828*::Tn*10*	Laboratory collection
TT172	*cysG1510*::Tn*10*	Laboratory collection
TT173	*cysJ1511*::Tn*10*	Laboratory collection
TT1674	*melB363*::Tn*10*	Laboratory collection
TT3698	*araE660*::Tn*10*	This work
TT3699	*ara-651*::Tn*10*	This work
TT12238	*his-10081*::Mud*F*	Laboratory collection
TT10738	*nadB499*::Mud*J*	Laboratory collection
TT11460	*metE2113*::Mud*J*	Laboratory collection
TT12304	*purF2054*::Mud*J*	Laboratory collection
TT13041	*cysG1573*::Mud*J*	Laboratory collection
TT14528	*cysA1585*::Mud*J*	Laboratory collection
TT14749	*pyrE2678*::Mud*J*	This work
TT14750	*aroE568*::Mud*J*	This work
TT14834	*cysJ1539*::Mud*J*	Laboratory collection
TT10270	*trp-3477*::Mud*K*	Laboratory collection

All strains are derivatives of *S. typhimurium* LT2.

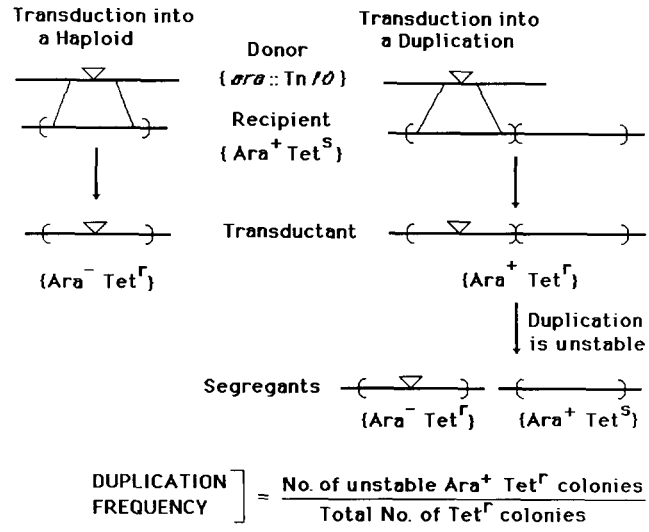

FIGURE 1.—Transductional cross for detecting duplications.

and ampicillin: 15 μg/ml and 30 μg/ml. L-Arabinose, L-malate, D-melibiose, 2,3,5-triphenyltetrazolium chloride and the antibiotics were obtained from Sigma Chemical Company. D-Sorbitol was obtained from Matheson, Coleman and Bell.

Growth in batch cultures: Overnight batch cultures of *S. typhimurium* LT2 were used initially to estimate the frequency of duplications in nutrient broth grown cultures. For estimating duplication frequency in minimal arabinose medium, the cultures were grown by serial subculture. Saturated cultures of *S. typhimurium* LT2 grown up in 10 ml of medium were diluted 1000-fold into 10 ml of fresh medium. Subcultures remained at saturation for 6 to 10 hr before being rediluted.

Growth in chemostats: The culture volume in the chemostat was set between 80 and 100 ml. All chemostat cultures were heavily aerated by forced air and rapid stirring to avoid any possibility of growth limitation by oxygen. Because of this aeration, population sizes of 8×10^9 to 1×10^{10} cells/ml are obtained when the concentration of carbon source in the chemostat reservoir is high (0.2%). The concentration of carbon source in the chemostat reservoir for experiments that are described below was either 0.02 or 0.03%. This concentration can support growth of a standard batch culture (in a gyrotory shaker) to a population of about 2×10^8 cells/ml. Population sizes in the chemostat were between 5×10^8 and 1×10^9 cells/ml. The cell generation times for individual chemostat experiments, described in the text, were calculated according to KUBITSCHEK (1970).

Samples were collected through the exit tube. Once every 72 hr the pH of the chemostat was monitored; samples were typically pH 6.85. Once every 72 hr, chemostat samples were tested to determine if growth in the chemostat was limited only by the availability of carbon source. The chemostat samples were filtered. The filtrate was divided into two aliquots. To one of the aliquots the carbon source was added to a concentration of 0.2%; additional carbon source was not added to the second aliquot. Both aliquots were inoculated with single colonies of *S. typhimurium* LT2 and incubated overnight at 37°. After overnight growth the cultures were inspected visually. The cultures to which the carbon source had been added grew to full density (approximately 2×10^9 cells/ml). There was no obvious growth in cultures to which the carbon source had not been added indicating that growth in the chemostat was limited only by the availability of carbon source.

In all of the chemostat experiments except one (discussed below), the chemostat cultures (80–100 ml) were inoculated using 1 ml of a culture of *S. typhimurium* LT2 grown to maximum density in the same medium as that in the chemostat reservoir. The onset of starvation in the chemostat was presumed to occur when the chemostat cultures attained a stable final density (5×10^8 to 1×10^9 cells/ml). This was about 10–12 hr after the inoculation of the chemostat culture. The one exception was a sorbitol-limited chemostat in which the average cell generation time was 5 hr. In this one case, the chemostat was inoculated with 90 ml of a batch culture of *S. typhimurium* LT2 grown in 0.03% sorbitol until the carbon source was exhausted. Because the chemostat culture was already starving at the start of the experiment and no excess volume of medium with nutrient was available, the onset of starvation in this chemostat was presumed to occur immediately.

Transductional methods: The high frequency generalized transducing mutant of bacteriophage P22 HT105/1 *int-201* (SCHMIEGER 1972) was used for all transductional crosses. In transduction crosses involving selection for tetracycline resistance, equal volumes of saturated cultures of recipient cells and donor lysates were mixed and incubated in liquid media at 37° for 20 min before spreading onto selective medium. Transductants were first purified selectively to prevent the loss of duplications by segregation. Phage-free colonies were then identified as light colonies formed following streaking on green indicator plates (CHAN *et al.* 1972). Phage-sensitive colonies were identified by cross streaking with P22 H5 (a clear plaque mutant of phage P22). In transductional crosses involving selection for kanamycin resistance, recipient cells and donor phage were preincubated nonselectively on solid medium for 16–24 hr before replica-printing onto selective plates. In some transduction crosses, transductants were obtained by cross-streaking recipient cells against donor phage lysates on selective plates (see *Endpoint mapping of duplications*).

Transduction assay for duplications: The presence of duplications that include *ara* genes was monitored by transduction crosses in which the strain to be checked is the recipient (Figure 1). The donor strain used carries an *ara*::Tn*10* insertion (KLECKNER *et al.* 1975). Strains to be tested were transduced to tetracycline resistance (Tc^r) on

nutrient broth plates. Transductant (Tc^r) colonies were replica-printed to minimal arabinose plates to score the ability to use arabinose as a carbon source (Ara^+). Cells which inherit the *ara*::Tn*10* insertion and remain Ara^+, must have two copies of the *ara* gene in question or are due to transposition of the Tn*10* insertion from the *ara* locus do some other site in the genome. Duplication-containing transductant strains can be verified because they are unstable and segregate haploid strains that are phenotypically either Ara^-, Tc^r or Ara^+, Tc^s. Therefore, representative samples of the potential duplications (Ara^+, Tc^r transductant colonies) were tested for stability of the Ara^+ and Tc^r phenotypes. The frequency in the original population of duplications that include the *ara* locus is defined as the fraction of the total Tc^r transductants that are both Ara^+ and show unstability.

A similar transduction assay was used for detecting the frequency of duplications that include the melibiose permease gene (*melB*). A Tn*10* insertion in the *melB* gene was transduced into recipient strains by selecting for Tc^r on melibiose-tetrazolium indicator plates. On these plates, strains that are able to use melibiose as a carbon source (Mel^+) form white colonies and strains that are unable to use melibiose as a carbon source (Mel^-) form red colonies. Duplication-containing cells that have inherited the *melB*::Tn*10* insertion in one copy of the duplication are Mel^+ and segregate Mel^- clones because the duplication is unstable. The colonies of these strains show a sectored appearance on melibiose-tetrazolium plates. The frequency of duplications that include the *melB* locus is defined as the fraction of the total Tc^r transductants that are sectored.

Endpoint mapping of duplications: Duplications were mapped by transduction crosses that are similar to the crosses used to demonstrate the presence of the duplication. The duplication strain was used as a recipient in crosses with a series of donor strains each having an auxotrophic insertion of the transposable element Mu*d-I-1768* (Mu*dJ*), which encodes kanamycin resistance (Kan^r) (CASTILHO, OLFSON and CASADABAN 1984). The Kan^r transductants were screened for inheritance of the donor auxotrophy. If the Kan^r colonies are prototrophic, the site of the Mu*d* insertion is inferred to lie within the duplicated segment. If the Kan^r colonies are auxotrophic, the site of the Mu*d* insertion is outside the duplicated segment. For one of the loci tested, a Mu*dF* insertion was used instead of Mu*dJ*. Mu*dF* is a transposition defective derivative of bacteriophage *Mu* that encodes kanamycin resistance and also includes the entire *lac* operon of *Escherichia coli* (CHACONAS *et al.* 1981); R. V. SONTI and J. R. ROTH, unpublished results). For another locus that was tested, a Mu*d-II-1768* (Mu*dK*) insertion which also encodes kanamycin resistance (CASTILHO, OLFSON and CASADABAN 1984), was used instead of Mu*dJ*.

A modification of this method was used to map duplication endpoints when a distinctive colony morphology was the only phenotype associated with the duplication. This method was used to map the endpoints of duplications carried by strains that were selected in a sorbitol-limited chemostat. These duplication strains, called Lsr^+II strains (see RESULTS for details), were identified on the basis of their distinctive colony morphology on minimal arabinose plates. These duplication strains are selected in a sorbitol-limited chemostat but are overgrown by haploid segregants when they are grown in batch cultures.

The difficulty of maintaining these strains made it impossible to map the duplication endpoints using the method described above. Therefore, colonies containing these duplications were picked directly from minimal arabinose plates and cross-streaked against donor phage on solid selective media. When strains with Tn*10* insertions in either *cysJ*, *argG* or *cysG* were used as donors in these transduction crosses, the Tc^r transductants remained prototrophic, indicating that the sites of these donor insertions lie within the region that is duplicated in the Lsr^+II strain (Prototrophic, Tc^r transductants were not obtained in control crosses for which *S. typhimurium* LT2 strain was used as the recipient). We can selectively maintain the duplication in the prototrophic, Tc^r transductants formed when a Tn*10* insertion in either *cysJ*, *argG* or *cysG* is transduced into the Lsr^+II strains. This makes it possible to characterize these duplications. The Tc^r, prototrophic transductant strains, still retain the distinctive colony morphology and growth properties that are characteristic of the parental Lsr^+II strain (see RESULTS). The extent of the duplication in the Lsr^+II strains was determined by using the prototrophic, Tc^r derivatives as recipients in transduction crosses with donor strains that have auxotrophic insertions of either Mu*dF*, Mu*dJ* or Mu*dK*.

Colony morphology: Colony morphology served as a useful means of scoring duplication types that recurred. Morphology was determined by inspecting colonies directly or with the aid of a dissecting microscope; in each case the correlation between a colony morphology and a particular genotype was verified by other means. The colony types scored are described in RESULTS.

Assay for duplications that improve growth on malate: Cells were plated onto minimal malate medium and the frequency of rapidly growing (Mte^+) strains was determined by counting large colonies. A representative sample of the Mte^+ strains was tested for stability of the Mte^+ phenotype by streaking cells on minimal malate medium. Duplication-containing strains form mostly large colonies, plus some small colonies formed by slow growing (Mte^-) haploid segregant cells.

RESULTS

It seems likely that enteric bacteria in nature are frequently starved for a carbon and energy source. Therefore we grew *S. typhimurium* under these conditions to determine whether duplications might be selectively valuable for adaptation of this bacterium. Initially arabinose was used as the limiting resource. Following growth on limiting arabinose, the frequency of duplications that include *ara* genes was determined. The two genetic regions tested are the *araBADC* locus at minute 2 and the *araE* gene at minute 63. The *araC* gene encodes the activator protein of the *ara* regulon and the *araBAD* genes encode degradative enzymes (LEE, NISHITANI and WILCOX 1984). The *araE* locus encodes the arabinose permease (LEE, AL-ZARBON and WILCOX 1981). It should be noted that *Escherichia coli* has two arabinose transport systems. The *araE* system, analogous to that of Salmonella, is a low affinity system. A high affinity system encoded by the *araFG* genes maps at minute 45 of the *E. coli* chromosome. The *araFG* system does not seem to be present in *S. typhimurium* (LEE *et al.* 1982).

Duplication frequency of *ara* genes: The frequency of cells with duplications of *ara* genes was estimated in cultures grown in nutrient broth using

TABLE 2

Duplication frequencies of *ara* utilization genes

Growth medium[a]	No. of generations	Frequency of duplications including *ara* operon (2 min) ($\times 10^{-4}$)	Frequency of duplications including *araE* gene (63 min) ($\times 10^{-4}$)
Nutrient broth	10	4	10
High arabinose (0.2%)	60	4	10
Low arabinose (0.01%) Expt. 1	60	Not tested	320
Low arabinose (0.01%) Expt. 2	60	4	10
Low arabinose (0.01%)	180	6	1830
Low arabinose (0.01%) Expt. 3	200	4	930
Low arabinose (0.01%) Expt. 4	200	15	300

[a] The growth protocol is described in MATERIALS AND METHODS.

the Tn*10* transduction method described in MATERIALS AND METHODS. These conditions should be non-selective for arabinose and reflect the basal duplication frequency. The duplication frequency of the *araBADC* locus (at minute 2) is 4×10^{-4}; the duplication frequency of the *araE* locus (at minute 63) is 10×10^{-4} (Table 2). These frequencies are typical of those found for most chromosomal loci (ANDERSON and ROTH 1981).

The effect of prior growth conditions on duplication frequency was then tested. Cells that had been grown on high (0.2%) arabinose for 60 generations showed no change in the duplication frequency of either of the *ara* loci (Table 2), suggesting that growth on a high concentration of arabinose does not select for duplications of either of these regions.

Growth on medium with limiting arabinose: To test the selective effect of growth on limiting arabinose, we grew *S. typhimurium* LT2 on medium with a low arabinose concentration (0.01%). With this initial concentration, cells grew to a final concentration of 1×10^8 cells/ml. By serially subculturing into the same medium we could test the selective effect over many generations.

During growth of batch cultures on medium with low arabinose (0.01%), there is a selection for cells that contain duplications of the arabinose permease gene (Table 2); under the same growth conditions there is no selection for cells with duplications of the *araCBAD* locus at minute 2. This experiment has been carried out several times and in all cases an increase in the frequency of *araE* duplications was observed. The maximum increase in duplication frequency was 183-fold following 180 generations of growth in low arabinose medium. The variation in individual determinations may be due to the fact that these experiments involved serial subculture of batch cultures that were held at saturation for different periods of time before being subcultured (see MATERIALS AND METHODS). There is a decrease in duplication frequency when cultures remain in saturation phase for prolonged periods of time (R. V. SONTI and J. R. ROTH, unpublished results). In later experiments (see below) this variation was avoided by growing cells in arabinose-limited chemostats.

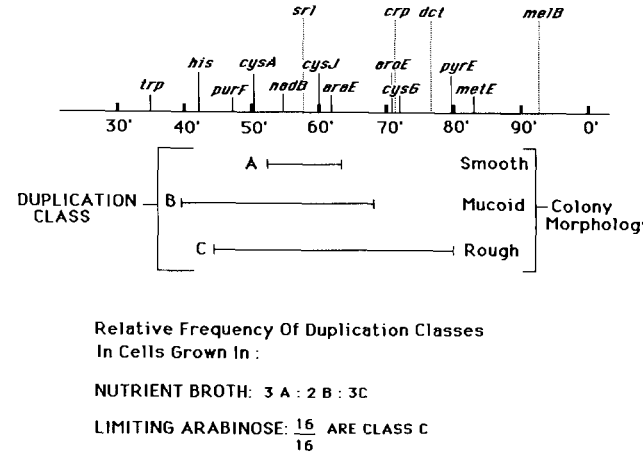

FIGURE 2.—Endpoint mapping of *araE* duplications. The horizontal line at the head of the figure indicates map position in minutes on the chromosome. The solid vertical lines that extend from this line indicate the auxotrophic loci that were tested for merodiploidy. In nutrient broth grown cultures 8 duplications that were isolated from one experiment were mapped. In limiting arabinose grown cells, 16 duplications that were isolated in two separate experiments were mapped. Segregation analysis of class A duplications indicates that one endpoint is very close to *araE* (R. V. SONTI and J. R. ROTH, unpublished results). The dotted lines indicate the map positions of some genes that are involved in carbon source utilization. These genes are discussed in the text.

Endpoint mapping of *araE* duplications: Spontaneous duplications of the *araE* locus that arose during non-selective growth in nutrient broth should represent the major classes of spontaneous duplications that include the *araE* locus. These duplications, shown in Figure 2, fall into three classes. Class A duplications extend from minute 52 to minute 63, class B duplications extend from minute 40 to minute 70 and class C duplications extend from minute 45 to minute 80. Strains containing each of these duplication classes have a unique colony morphology on minimal arabinose plates. Class A duplication strains form smooth colonies; class B duplication strains form mucoid colonies and class C duplication strains form rough colonies. In nutrient broth grown cultures, all three duplication types are present in equal proportions (Figure 2).

The initial mapping of endpoints and the correlation of endpoints with colony morphology was done using data from one nutrient broth culture. The frequency of *araE* duplications was then determined again for several other nutrient broth cultures, using colony morphology to score duplication types. In these later experiments only a few duplications were mapped in detail (R. V. SONTI and J. R. ROTH, unpublished results). All data are consistent with the results presented in Figure 2.

Endpoint mapping of *araE* duplications selected in limiting arabinose: Duplications selected during growth on limiting arabinose were isolated by the Tn*10* transduction method and their endpoints were mapped. All of the 16 duplications tested have endpoints characteristic of class C duplications (Figure 2). These 16 duplication strains all show the rough colony morphology characteristic of class C duplications. Several hundred other duplication strains isolated from low arabinose medium by the Tn*10* transduction method have been examined and all of these strains also exhibit the rough colony morphology characteristic of class C duplications. These results show that selection for improved growth in low arabinose medium appears to favor only class C duplication strains. Duplication strains selected during growth on limiting arabinose medium will be referred to as Lar^+ strains.

Lar^+ duplication strains are similar to duplication strains that grow rapidly on malate: The duplications in the Lar^+ strains include the same region of the chromosome as previously described duplications that permit rapid growth on minimal malate medium (STRAUSS and HOFFMANN 1975). *S. typhimurium* grows poorly on minimal medium with L-malate as the sole source of carbon and energy. Spontaneous mutants that grow rapidly on minimal malate medium (Mte^+) arise at a frequency of 1×10^{-3} to 1×10^{-4} when nutrient broth grown cultures of *S. typhimurium* LT2 are plated for single colonies on minimal malate medium. The vast majority of these Mte^+ mutants carry duplications which extend from minute 45 to minute 80 on the *S. typhimurium* chromosome. These results were originally obtained by STRAUSS and HOFFMANN (1975) and were repeated by us. The frequency of Mte^+ duplication strains in nutrient broth grown cultures (1×10^{-3} to 1×10^{-4}) is similar to the frequency of class C duplications in nutrient broth grown cultures (Table 2; Figure 1). Also, Lar^+ and Mte^+ duplication strains have a similar colony morphology on both minimal arabinose and minimal malate media.

Because of the similarities between the two duplication types, we tested the possibility that the duplications selected by growth on limiting arabinose might confer improved growth on malate. Sixteen Lar^+ duplication strains and three class C duplication-containing strains isolated from nutrient broth grown cultures were streaked for single colonies on minimal malate medium. All of these 19 strains form large colonies on minimal malate medium indicating that they possess the Mte^+ phenotype. All of these duplication-containing strains also segregate slow growing (Mte^-) strains that give rise to small, smooth colonies on minimal malate medium. The Mte^- strains are presumably haploid segregants in which the duplication has been lost by recombination. These results indicate that the Lar^+ duplication strains and the Mte^+ duplication strains carry very similar, or identical, duplications. Thus, one duplication can confer a fast growth phenotype for two different limiting carbon sources.

The common factor in the two selection conditions used above is that growth is limited by the availability of a carbon and energy source and that the permease genes for the two carbon sources are included within the chromosomal segment that is duplicated (KAY and CAMERON 1978; LEE, AL-ZARBON and WILCOX 1981). Results are presented later in this manuscript to show that the same duplication is also selected in sorbitol-limited chemostats; the permease gene for sorbitol is also included within the same duplicated segment. This duplication is not selected on limiting melibiose, a sugar whose permease maps elsewhere on the chromosome.

Selection for *araE* duplications in arabinose-limited chemostats: Chemostats are devices in which bacteria can be continuously cultured for long periods of time under conditions in which growth is limited by the concentration of a particular nutrient (NOVICK and SZILARD 1950; MONOD 1950; DYKHUZIEN and HARTL 1983). They have been used extensively to study differential growth rates between strains (for review see DYKHUZIEN and HARTL 1983). In chemostat experiments the selection for *araE* duplications was assayed by use of the Mte^+ phenotype. As described earlier, only class C duplications (not class A or B) are selected in arabinose-limited batch cultures and these class C duplications also cause rapid growth on malate. Therefore the selection for arabinose permease duplications within the chemostat can be followed by plating cells at regular intervals onto minimal malate medium and measuring the frequency of Mte^+ colonies. Representative samples of the Mte^+ strains, thus obtained, were picked at each time point and tested for colony morphology on minimal arabinose medium and for stability of the Mte^+ phenotype.

Chemostats were run at two different doubling times. A strong selection for *araE* duplications was observed under both of the conditions that were tested (Figure 3). In arabinose-limited chemostats with an average cell generation time of 2.3 hr, the permease duplication frequency increases until, after 74 hr in the chemostat, they constitute 96% of the population. This represents a 3800-fold increase in the frequency of these duplications in the course of 32 doublings of the chemostat culture. In chemostats in which the average cell generation time is 16 hr, the duplication frequency is 52% after 42 hr in the chemostat. This represents a 1600-fold increase in the frequency of permease duplications. During this period, the chemostat culture has doubled only 2 to 3 times, showing that under conditions of extreme starvation there is a very strong selection for duplica-

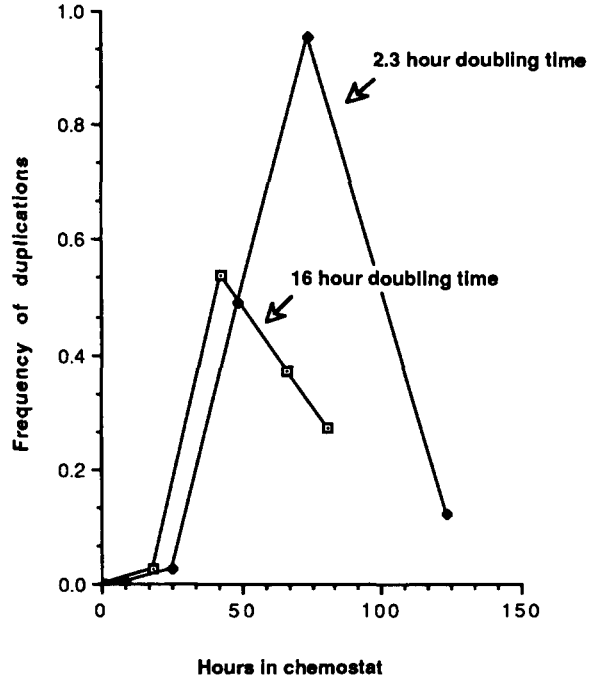

FIGURE 3.—Frequency of *araE* duplications in arabinose-limited chemostats. Duplication frequency was estimated by plating cells onto minimal malate medium and determining the frequency of Mte^+ duplication strains. For each doubling time, results from one chemostat experiment are presented. Similar results were obtained, for each doubling time, in independent chemostat experiments.

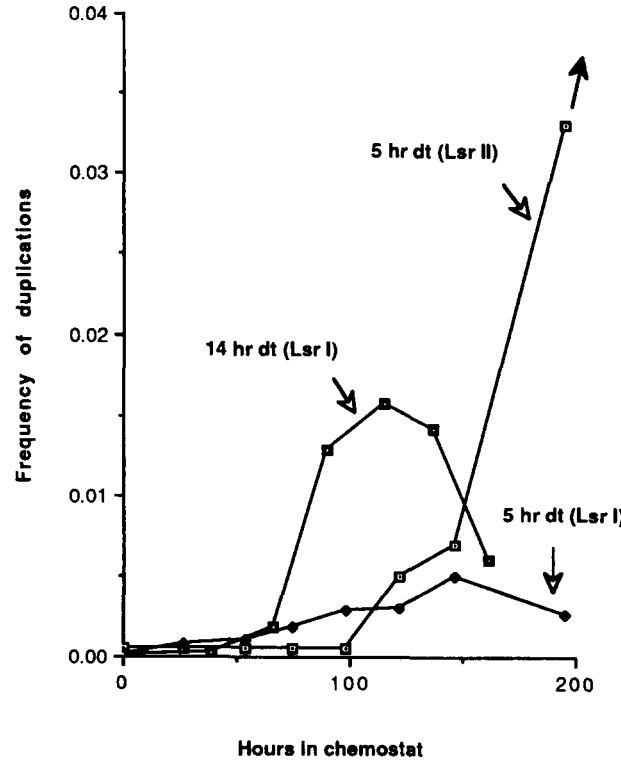

FIGURE 4.—Frequency of duplication strains in sorbitol-limited chemostats. *S. typhimurium* was grown in sorbitol-limited chemostats with an average cell generation time of either 5 hr or 14 hr. The sorbitol limited chemostat in which the cell generation time is 5 hr was inoculated using a protocol which is different from that used for the other chemostat experiments described in this manuscript (MATERIALS AND METHODS). The Lsr^+I and Lsr^+II duplications arose sequentially in the chemostat with a 5 hr doubling time. These duplications show the same endpoints, but Lsr^+II duplications do not shown an Mte^+ phenotype. The frequency of Lsr^+II duplications increases (as shown by solid arrow) during continued growth on limiting sorbitol (see RESULTS). dt = doubling time and hr = hours.

tion-containing cells and suggesting that most of the growth under these conditions is due to the duplication-containing fraction of the population. Under both growth conditions, the frequency of duplications decreases after reaching a peak value. We presume that this is due to the appearance of faster growing mutants which displace the duplication-containing strains.

Selection for duplication strains in sorbitol-limited chemostats: To test the generality of the duplication phenomenon, we tested sorbitol, another sugar whose permease maps within the chromosomal segment that is duplicated in the Mte^+ strains (SANDERSON and ROTH 1988). Figure 4 shows that the frequency of Mte^+ duplications increases in sorbitol-limited chemostats. There is a 25-fold increase in the frequency of such duplications after 137 hr (27 doublings) in a sorbitol-limited chemostat in which the average cell generation time is 5 hr. The response of duplication frequency to this selection is not as large as that on limiting arabinose or malate media but it is significant. These results indicate that the same duplications that confer the Mte^+/Lar^+ phenotype are also selected in limiting sorbitol medium. The selection on limiting sorbitol media is referred to as the Lsr^+ phenotype; these results show that the selected Lsr^+ duplications also show the Mte^+ phenotype. The endpoints of the duplications in five of the Mte^+/Lsr^+ strains were determined. The Mte^+/Lsr^+ duplication strains are merodiploid for the chromosomal segment between minute 45 and minute 80. The endpoints of the duplication in the Mte^+/Lsr^+ strains are similar to the endpoints of the duplications in the Mte^+/Lar^+ strains that were described above. These results show that very similar or identical duplications are present in the two kinds of strains and suggest that the Mte^+/Lar^+ strains are also Lsr^+.

In the sorbitol selection (5-hr doubling time, Figure 4), the frequency of Mte^+ duplications increased to a maximal value and then decreased. As the Mte^+ duplication strains decreased in frequency, another duplication-containing strain increased in frequency within the chemostat (Figure 4). After 196 hr (39 doublings) of growth in the chemostat there is at least a 200-fold increase in the frequency of these secondary duplication types and they constitute 3.5% of the population in the chemostat. This duplication strain will be called the Lsr^+II strain because it is the second class of duplication containing strains that are selected in limiting sorbitol medium. The frequency of Lsr^+II strains increases in this chemostat until after 220 hr

(44 doublings) the Lsr$^+$II strains constitute 16% of the chemostat population.

The Lsr$^+$II duplication strains were identified by their rough colony morphology on minimal arabinose media and their frequency within the sorbitol limited chemostat was assayed by plating chemostat cultures on minimal arabinose plates. The Lsr$^+$II strains have a rough colony morphology on minimal arabinose plates and are unstable for this rough colony phenotype. Endpoint mapping reveals that all of the seven Lsr$^+$II strains tested are merodiploid for the chromosomal segment between minute 45 and minute 80 (see MATERIALS AND METHODS for details about endpoint mapping). However, they are different from the Mte$^+$/Lsr$^+$ duplication strains because they grow slowly and form small colonies on minimal malate medium (Mte$^-$).

The endpoint mapping experiments indicate that the Lsr$^+$II strains have endpoints that are similar to the endpoints of duplications in strains that are Mte$^+$/Lsr$^+$. However, the fact that the Lsr$^+$II strains are Mte$^-$ shows that these duplication-containing strains are different from the Mte$^+$/Lsr$^+$ strains. The duplications could have slightly different endpoints indistinguishable by the mapping technique that has been employed. Alternatively, it is possible that the duplications have identical endpoints but that the Lsr$^+$II strains have an additional mutation that provides a selective advantage on limiting sorbitol and also causes the strains to be Mte$^-$. The exact nature of the difference between the Lsr$^+$II strains and the Mte$^+$/Lsr$^+$ strains remains to be determined.

The selection for the Mte$^+$/Lsr$^+$ duplication strains was also examined in a more extreme sorbitol-limited chemostat, in which the average cell generation time was 14 hr (Figure 4). After 115 hr (8 doublings) in this chemostat, the Mte$^+$/Lsr$^+$ duplication strains form 1.5% of the chemostat population. This represents a 160-fold increase in the frequency of the Mte$^+$/Lsr$^+$ duplication strain. After this point, the frequency of this duplication strain in the chemostat population decreases. Also, there is a simultaneous increase in the frequency of duplication strains that exhibit colony morphology (rough on minimal arabinose) and growth properties (Mte$^-$) that are characteristic of the Lsr$^+$II duplication strains (R. V. SONTI and J. R. ROTH, unpublished results).

The Mte$^+$/Lsr$^+$ duplication strains are selected more strongly in a sorbitol-limited chemostat in which the average cell generation time is 14 hr compared to the chemostat in which the average cell generation time is 5 hr (Figure 4). This could be because the selection for the duplication is more stringent under conditions of greater starvation or because of a difference in the manner in which the two chemostat cultures were inoculated (see MATERIALS AND METHODS).

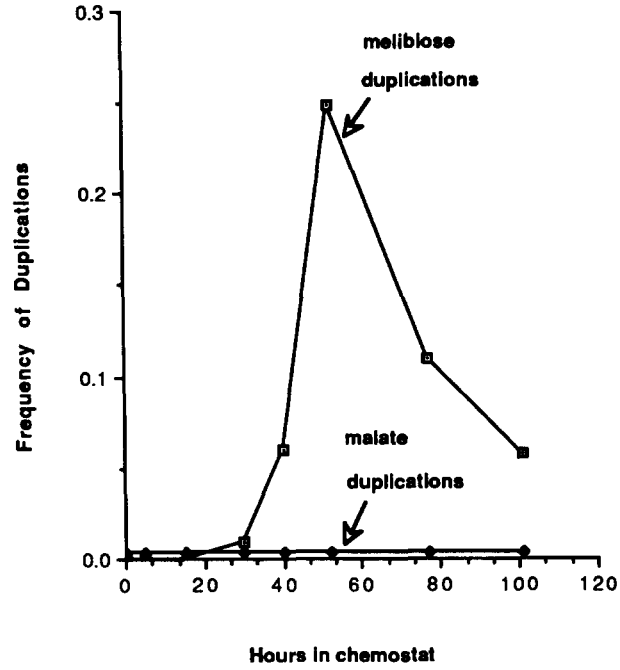

FIGURE 5.—Frequency of melibiose permease gene (*melB*) duplications and duplications that confer rapid growth on malate (Mte$^+$) in a melibiose-limited chemostat in which the average cell generation time is 18 hr (see MATERIALS AND METHODS and RESULTS for details about the assays).

However, these results show that the Mte$^+$/Lsr$^+$ duplication strains are reproducibly selected in sorbitol-limited chemostats.

Selection for duplication strains in melibiose limited chemostats: The permease genes for arabinose, malate and sorbitol are encoded within the duplications in the strains that were selected in the experiments described above. The melibiose permease is encoded by the *melB* locus which maps at minute 93 on the *S. typhimurium* chromosome (SANDERSON and ROTH 1988), outside the duplication found in the Mte$^+$ strains. Therefore we tested for the appearance of Mte$^+$ duplications in melibiose-limited chemostats.

The results presented in Figure 5 show that there is no selection for the Mte$^+$ duplication strain in a melibiose-limited chemostat in which the average cell generation time is 18 hr. The frequency of the Mte$^+$ duplication strain in this melibiose limited chemostat was approximately 4×10^{-4} at all time points (Figure 5). Similar results were found for a chemostat with a 26-hr generation time (R. V. SONTI and J. R. ROTH, unpublished results). These results, when considered with the fact that the *melB* locus is not duplicated in the Mte$^+$ strain, suggest that the Mte$^+$ duplication strains are selected during growth on a limiting carbon source only if the permease gene for the carbon source is duplicated in the Mte$^+$ strain.

The melibiose-limited chemostats were tested for the accumulation of duplications that do include the melibiose permease gene (*melB*). The frequency of *melB* duplications was estimated using the Tn*10* trans-

duction assay described in MATERIALS AND METHODS. The results presented in Figure 5 show that there is a strong selection for strains with duplications of the *melB* locus in a melibiose-limited chemostat with an 18-hr doubling time. After 52 hr (3 doublings) in this chemostat, there is a 1300-fold increase in the frequency of cells with duplications of the *melB* locus. At this point *melB* duplication-containing cells constitute 25% of the chemostat population. These results indicate that most of the growth in the chemostat is due to the fraction of the population that contains *melB* duplications. After reaching a peak value, the frequency of duplication containing cells in the population decreases, presumably because fitter mutants are displacing the duplications from the chemostat. Similar results were obtained in a chemostat at 29 hr doubling time (R. V. SONTI and J. R. ROTH, unpublished results).

DISCUSSION

The results presented here show that duplication-containing cells are selected under conditions of growth limitation by the availability of a carbon and energy source. Under the most stringent selective conditions in chemostats, most of the growth in the population is occurring within the duplication containing fraction of the population. This selection continues until the duplications are displaced from the chemostat, presumably by other variant strains that are better adapted for growth within the chemostat.

The average doubling time for enteric bacteria in nature has been estimated to be about 40 hr (SAVAGEAU 1983). If this estimate is correct, bacteria must spend a considerable amount of time under conditions of stringent growth limitation. It is generally believed that scarcity of nutrients is a major factor in causing these slow doubling times. The chemostat experiments show that a particular class of duplications has a huge selective advantage during similar starvation conditions. These results indicate that under natural conditions duplication-containing strains would have a similar selective advantage and that they could play a major role in bacterial adaptation to stressful situations brought about by starvation conditions. Duplication-containing cells would be particularly useful under fluctuating environmental conditions because a duplication can be lost by segregation when environmental conditions change; thus the advantageous variant is not committed to its new genotype when selective conditions no longer favor that duplication. In contrast, a stable mutation selected under one set of conditions might prove to be deleterious under other circumstances. There is some reason to believe that the growth of enteric bacteria is exactly this alternating series of periods of starvation and periods of rapid growth (KOCH 1971).

S. typhimurium strains with duplications of the chromosomal segment between minute 45 and minute 80 are selected over the parent haploid strain on limiting arabinose, malate and sorbitol media. The permease genes for these sugars are encoded within this chromosomal segment (Figure 2). The permease gene for melibiose maps at minute 93 and is located outside the chromosomal segment that is duplicated in the Mte^+ strains. There is no selection for this duplication in a melibiose-limited chemostat but under the same conditions there is a strong selection for other duplications that include melibiose genes. These results suggest that the selection for the Mte^+ duplication strains may exist because they are more efficient for uptake of the sugars. The chromosome segment between minute 45 and minute 80 encodes the permease genes for several other compounds that can serve as sources of carbon and energy for *S. typhimurium* (SANDERSON and ROTH 1988). Therefore, selection for the Mte^+ duplication may exist on limiting concentrations of several other carbon sources besides arabinose, malate and sorbitol. This property of the duplication-containing cell may be particularly advantageous under conditions in which a mixture of compounds that can serve as a carbon and energy source is available or when the composition of the carbon source is changing rapidly with time.

Duplication of the permease gene may be necessary but it is probably not sufficient to confer the selective advantage seen here. Only class C duplications are selected on limiting arabinose; the class A and class B duplications, which also include the *araE* gene, are not selected. Thus only class C duplication strains confer a growth advantage even though all three duplication types are represented at approximately equal frequencies under nonselective conditions. This suggests that some gene or genes that are included in class C duplications but not included in either class A or class B duplications contribute to the selective advantage conferred by the class C duplication.

The *crp* locus which encodes the catabolite activator protein and maps at minute 73 on the *S. typhimurium* chromosome (SANDERSON and ROTH 1988) is included in the class C duplications but not included in class A or B duplications (Figure 2). The catabolite activator protein is required for the optimal expression of a number of genes that encode proteins that are involved in the utilization of carbon sources (ULLMANN and DANCHIN 1983). The duplication of the *crp* locus may contribute to the selective advantage conferred by class C duplications and explain why neither class A nor B duplication strains are selected on limiting arabinose medium. Mutational analysis of Mte^+ duplication-containing strains indicates that duplication of the *crp* locus contributes to the selection for these strains on minimal malate medium (R. V. SONTI and

J. R. ROTH, unpublished results). This is consistent with the possibility that duplication of the *crp* locus may contribute to the selection for duplication-containing strains on limiting arabinose medium. Several other genes that are included in class C duplications along with the *araE* and *crp* loci may also contribute to the growth advantage conferred by this duplication on limiting arabinose.

The results discussed above suggest that duplications may be selected because they amplify multiple functions which contribute synergistically to improved growth. As seen here, the growth advantage provided by a duplication may be greater than the 2-fold that might be expected for an extra copy of the permease. This growth advantage is particularly impressive in view of the fact that the duplications are inherently unstable and frequently revert to the haploid state. It is likely that the simultaneous duplication of multiple, interactive functions can generate the unexpectedly strong selections for duplication-containing strains that have been observed in these experiments.

If the duplication of suitably interactive sets of genes is of great selective advantage to bacteria, one might predict that the map location of genes might be shaped by the desirability of clustering interactive sets of genes. The chromosome segment between minute 45 and minute 80 on the *S. typhimurium* chromosome appears to contain a set of genes, whose duplication might help *S. typhimurium* adapt to growth limitation brought about by carbon and energy starvation. Other chromosome segments might contain similar sets of interactive genes whose duplication could be advantageous under limiting conditions for nitrogen, phosphate, sulphate or other nutrients. Strains containing chromosome rearrangements such as inversions or transpositions that alter gene order might be counterselected in nature because they disrupt these gene sets whose duplication is occasionally advantageous.

The tests for merodiploidy that we have used do not distinguish between simple duplications and triplications or further amplifications. The presence of further amplifications beyond the merodiploid state may also explain the greater than 2-fold growth advantage for the duplication containing strains. It seems unlikely that cells would be selected with high order amplifications of this large chromosomal segment (minute 45 and minute 80). This segment is 30% of the total chromosome; it seems likely that the energetic cost of maintaining and replicating many copies of such a large segment would become prohibitive. However, it may be possible that further amplification of selected regions within this chromosomal segment occurs in the duplication-containing strains and that this may explain in part the unusually strong selections we have observed.

Duplications that are indistinguishable from the Mte^+ duplications described above have also been isolated under a completely different selection condition (HOFFMANN *et al.* 1983). The *aroC321* mutation of *S. typhimurium* causes auxotrophy for tryptophan, tyrosine and phenylalanine, but reverts to tryptophan prototrophy at a high frequency. All of these Trp^+, Tyr^-, Phe^- revertants are phenotypically Mte^+ and carry a duplication of the chromosomal segment from minute 45 to minute 80. The Trp^+ phenotype of these revertants is attributed to amplification of the partially defective *aroC* gene which lies within the duplicated segment (HOFFMANN *et al.* 1983). It seems likely that the Trp^+ phenotype requires amplification of more than just the *aroC* gene. All of the Trp^+ revertants were of the Mte^+ type; data reported here and by ANDERSON and ROTH (1981) demonstrate that other sets of *aroC* duplications arise at a high frequency. Apparently none of these other duplications confer a Trp^+ phenotype on the *aroC321* mutant.

According to current theory, the requirements for the formation of bacterial gene duplications are a functional recombination system and the presence of repeated sequences in the genome (ANDERSON and ROTH 1981; LIN, CAPAGE and HILL 1984). If duplications of the type described above are frequently advantageous in nature, it would represent a selective force acting to favor organisms in which advantageous gene sets are flanked by directly repeated sequences which can support recombination and thereby generate a duplication of the gene set. Site specific recombination systems for the formation of duplications may also be expected. Most, if not all, bacterial species for which data is available contain functional recombination systems and also carry repeated sequences in their genome. This makes it possible that gene duplications are being formed in all bacterial species. Adaptation by duplication formation may be a general feature of bacteria and other single-celled organisms that are faced with starvation conditions in nature.

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