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DNA Amplification and Rearrangements in Archival *Salmonella enterica* Serovar Typhimurium LT2 Cultures

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Variations in genome size and gene order were observed in archival *Salmonella enterica* serovar Typhimurium cultures stored for over 40 years. In one strain, microarray analysis revealed a large, stable amplification. PCR analysis of the same strain revealed a genomic duplication that underwent a translocation. Other strains had smaller duplications and deletions. These results demonstrate that storage in stabs over time at room temperature not only allows for further bacterial growth but also may produce an environment that selects for a variety of mutations, including genomic rearrangements.

Several investigators (3, 4, 8–10, 15, 16, 26) have examined genetic changes among survivors within populations of cells present in a harsh environmental habitat. In addition to the jettisoning of "excess baggage" genes, as postulated by Koch (17), it might be anticipated that certain genetic rearrangements, including amplifications, could increase the fitness of cells in the population. We have a unique set of several thousand bacterial samples that have experienced unfavorable growth conditions during 4 decades of storage in the presence of limiting nutrients and increasing metabolic by-products.

This extensive collection of *Salmonella enterica* serovar Typhimurium LT2 auxotrophic mutants accumulated by Miloslav Demerec and associates decades ago was the basis of inter- and intragenic chromosomal mapping (5–7). Upon inoculation over 4 decades ago, low reversion rates of less than 1 in 10^8 cells were recorded, indicating the stability of the auxotrophic phenotype upon inoculation into stabs. To date, 421 of these stabbed cultures that had been stored at room temperature have been opened, and each yielded colony formers, suggesting survival strategies that are currently not well understood.

Tests performed on 14 serovar Typhimurium LT2 strains included DNA-DNA hybridization analysis to compare the archived strains with wild-type serovar Typhimurium LT2 and PCR analysis to observe any change in genomic rearrangements at rRNA (*rrn*) operons. Five of these are listed in Table 1.

While nine of the strains had no detectable genomic anomalies, a few strains had relatively small chromosomal amplifications and deletions. Interestingly, one strain had a large-scale genomic rearrangement and amplification. Note that these assays were performed on LT2 strains that do not have mutator phenotypes (data not shown). Archival serovar Typhimurium LT7 strains, which have a mutator phenotype (19), have previously been shown to have undergone genomic rearrangements (8).

Previous work has shown that chromosomal duplications can occur at relatively high frequencies in the presence of limited carbon sources but that these duplications typically are not maintained for long periods of time once the strains are grown in an abundance of the previously limited nutrient (28). Additionally, other investigators have shown that stable genomic rearrangements due to recombination at rRNA (*rrn*) operons are frequently found in natural isolates of *Salmonella enterica*, but only in serovars which are host-specific pathogens (20). In stark contrast, natural isolates of non-host-specialized *Salmonella* serovars such as serovar Typhimurium have consistently been shown to have very conserved genomic arrangements (21). Recently, Liu et al. demonstrated that some archival strains of serovar Typhimurium have undergone genomic rearrangements at *rrn* operons, but only in LT7-derived strains, which are natural mutator strains (19).

Contrary to these documented results with LT7 mutator strains, we found that the genomic amplifications and translocations are stable following growth on rich media after their revival from the archive. In addition, rearrangements that have never previously been observed for serovar Typhimurium strain LT2 isolates were detected. Further analysis of these strains may elucidate survival strategies utilized by *Salmonella* in environments not conducive to optimal growth, such as exhaustion of nutrients and perhaps reduced O_2 availability.

MATERIALS AND METHODS

Strain revival. The selection of the strains used for this study was based on previous observations of phenotypic variables among survivors, as noted in Table 1 (8, 29, 30). Vials from the original sealed agar stab cultures (0.5 ml/agar stab vial) had been opened, and 0.5 ml of buffered saline was added. The agar-saline mix was thoroughly mashed, and the mixture remained at room temperature for 30 min so that large particles would settle and allow additional cells to leach out of the agar. Aliquots of supernatant were then streaked for single colonies on Luria-Bertani agar. Individual colonies were picked and inoculated into Luria-Bertani broth. The colony morphologies of these revived strains were relatively similar throughout this study. Stock cultures were prepared from single colonies for the reported phenotypic studies (8, 29, 30). Strains that had previously shown variation via pulsed-field gel electrophoresis (PFGE) of rare-cutting endonuclease (*Avr*II) digests (8) were chosen for further study using microarray and *rrn* PCR.

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TABLE 1. Genomic changes found in archival strains

Strain no.	Microarray finding(s)	Auxotrophy[a]; other characteristic(s) (reference)
1598	Deletion of STM1842-1844, including the *htpX* heat shock protein, and STM3883 (*rbsC*) to STM3886 (*rbsR*), a ribose transport operon	*hisD141*
1601	Amplification between STM2154 (*mrp*) and STM2168 (*pbpG*)	*hisF144*; *rrnD* is not detected in PCR assay
1656	180-kb amplification between STM3993 and STM4131, between two rRNA clusters; deletion of Gifsy-1	*thyA68*; extra *Xba*I band with PFGE (8)
1674	Deletion of Gifsy-1	*hisD2550*; low in HPI and HPII hydroperoxidases; defect in RpoS (29)
1700	Deletion between STM1225 and STM1226 (*potAB* operon)	*thyA314*; low in HPII hydroperoxidases; defect in RpoS (29)

[a] These auxotrophies were present upon the initial storage of the strains and were still present after approximately 4 decades in storage.

Microarray analysis. The development of a serovar Typhimurium LT2-specific DNA-DNA microarray hybridization chip (24, 25) allowed us to compare the genomes of cells from a set of archival strains with that of the sequenced parental strain (24). This chip consists of PCR-amplified whole open reading frames (ORFs) representing 4439 of the 4607 genes annotated in the serovar Typhimurium LT2 genome, generated with primers complementary to the 3′ and 5′ termini of the genes. In addition, 106 of the 109 ORFs of the serovar Typhimurium LT2 virulence plasmid pSLT were also amplified. PCR products were dried, subsequently resuspended in NCP buffer (45 mM sodium citrate, 330 mM NaCl, and 2 μg of proteinase K/ml [pH 7.0]), and spotted in triplicates onto SuperAmine-coated glass slides (TeleChem International, Sunnyvale, Calif.) by using the Omnigrid spotter (Genemachines, San Carlos, Calif.).

The methods for labeling of genomic DNA for microarray hybridization, data acquisition, and microarray data analysis were described previously (23). Briefly, genomic DNA for comparative genomic hybridizations was prepared from overnight cultures of serovar Typhimurium LT2 and 14 archival *S. enterica* serovar Typhimurium isolates by using the DNEasy kit (Qiagen, Valencia, Calif.). These archive strains were chosen because previous studies had yielded interesting observations of genetic diversity (8, 29, 30). The harvested nucleic acid was labeled by using Cy3- and Cy5-dCTP (Amersham, Piscataway, N.J.) and Klenow enzyme, and labeled DNA from strain LT2 and a query strain was hybridized to the chip at 62°C overnight. Subsequently, the chip was washed and scanned by using the ScanArray 5000 laser scanner (Packard BioChip Technologies, Billerica, Mass.) with ScanArray 2.1. Signal intensities were quantified with the QuantArray 2.0 software package (Packard BioChip Technologies). Two hybridizations were performed for each strain comparison with reciprocally labeled DNA from LT2 and the query strain. Since the array was spotted in triplicates, these hybridizations resulted in six data points per gene. For normalization, the contributions of each spot to total signal in both channels were calculated, and ratios of these contributions (query strain/Typhimurium LT2) were determined. Finally, the medians of the six ratios obtained for each spot were determined and plotted in the order of the genes on the Typhimurium LT2 genome. Spots resulting in low signal with Typhimurium LT2 genomic DNA (lowest 5%) were excluded from graphical representation.

PCR to determine *rrn* arrangement. PCR was performed with strains to determine the *rrn* arrangement within the genome of *Salmonella* strains. Briefly, oligonucleotide primers were designed to anneal to the DNA directly adjacent to the 5′ and 3′ ends of each of the seven *rrn* operons, generating a total of 14 primers. To test all possible *rrn* arrangements, 49 PCRs were performed. A PCR product of approximately 7 kb generated from a particular primer set indicates which *rrn* operons are present. Primer sequences and the PCR protocol are described in detail by Helm and Maloy (14). Some strains were checked for all possible *rrn* arrangements sets, while others were checked only for the presence of the standard seven *rrn* operons found in all natural serovar Typhimurium isolates to date (Fig. 1A).

RESULTS

Microarray results. Five of the thirteen strains tested by microarray analysis displayed genomic aberrations compared to the Typhimurium LT2 reference genome. Strain 1700 was found to have a deletion in the *potAB* operon (STM1225-1226). In addition, two regions of strain 1598 involving STM3883-STM3886 (a ribose transport operon) and

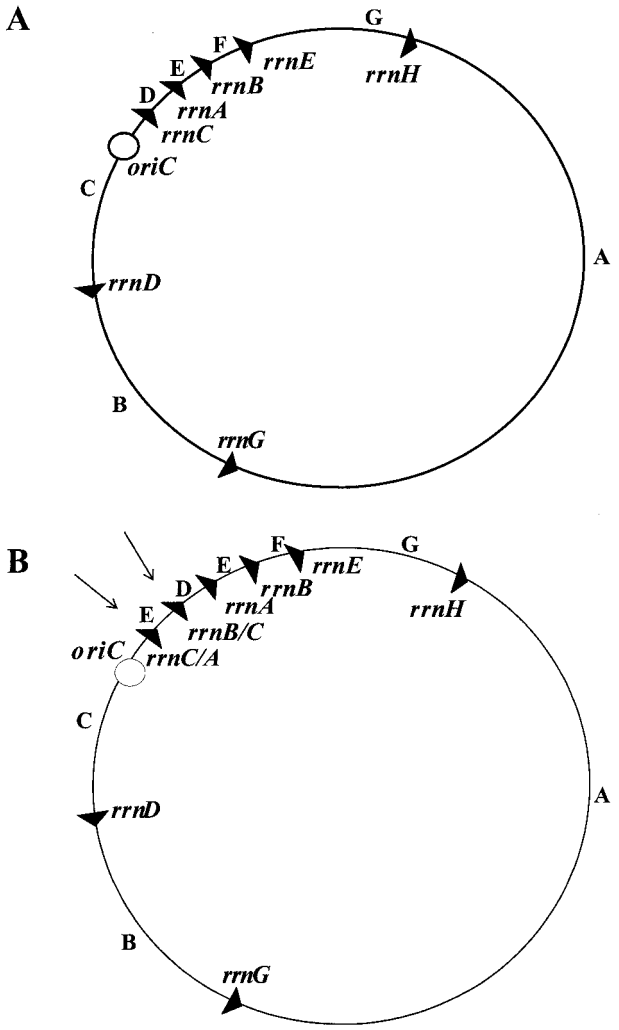

FIG. 1. (A) The *rrn* arrangement of *Salmonella enterica* serovar Typhimurium strain LT2. This arrangement is the same as that of *E. coli*, most other *Salmonella* serovars, and every natural isolate of serovar Typhimurium analyzed to date. Genomic regions between each *rrn* operon are designated I-*Ceu*I fragments. They are labeled A through G. The largest region, I-*Ceu*I fragment A, is approximately 50% of the genome, while the smallest region, I-*Ceu*I fragment F, at 44 kb is only 1% of the genome. (B) The *rrn* arrangement of strain 1656, based on PCR. The arrows indicate the hybrid *rrn* operons generated by the integration of the duplicated I-*Ceu*I fragment E into *rrnC*, yielding a total of eight *rrn* operons.

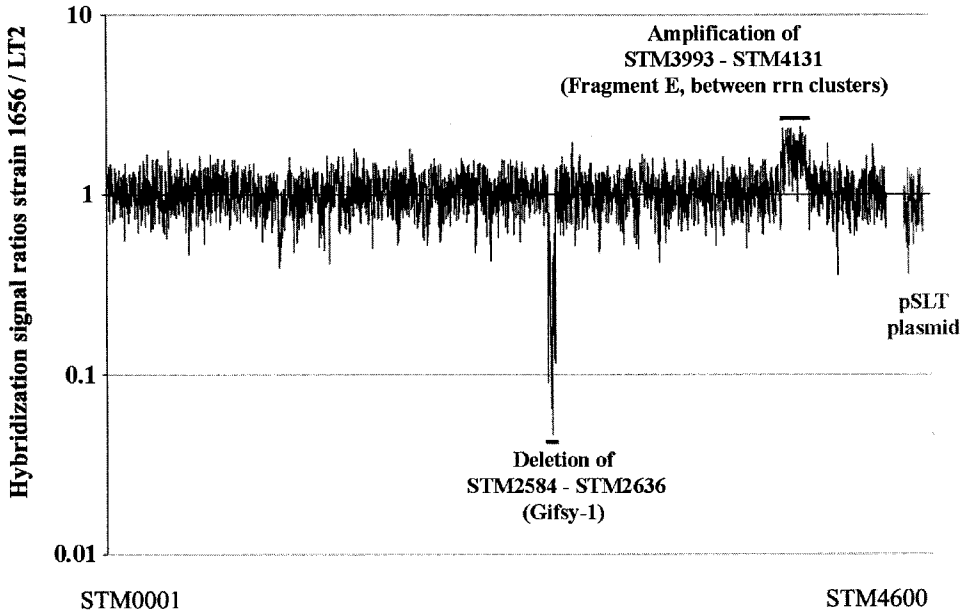

FIG. 2. Ratios of fluorescent signal intensities of strain 1656 over those of wild-type serovar Typhimurium LT2. Ratios are plotted in single-gene resolution in the order of the ORF position on the LT2 genome from left to right, starting at gene STM0001 and ending at gene STM4600. In addition, the ratios obtained for the pSLT virulence plasmid genes are depicted on the very right. Genes that resulted in weak fluorescent signal with LT2 genomic DNA (lowest 5%, i.e., 227 genes) were excluded from graphical representation. The large decrease in signal between STM2584 and STM2636 indicates a deletion in strain 1656. This region is the site of the prophage Gifsy-1 in wild-type serovar Typhimurium LT2. The increase in signal between STM3993 and STM4131 indicates a duplication relative to LT2. This region is the I-*Ceu*I fragment E, which is bordered by *rrn* operons.

STM1842-1844, which includes the heat shock protein gene *htpX*, were apparently deleted. An apparent amplification of the region between STM2154 and STM2168 was detected in strain 1601, and a sugar transport system, as well as both Fels prophage genomes, was deleted from strain 1674. Prophage sites, such as Fels-1 and Fels-2 and Gifsy-1 and Gifsy-2, were readily deleted from strains. The precise location of these sites can be found at http://www.ncbi.nlm.nih.gov/genomes/MICROBES/Complete.html.

Additionally, strain 1656 had a large amplified genomic region comprising about 4% of the entire chromosome, shown in the histogram in Fig. 2. Analysis of the other strains did not reveal any major deletions or amplifications.

PCR at *rrn* operons. Rearrangements in the *rrn* region were unexpected. Liu and Sanderson had noted that natural isolates of serovar Typhimurium from diverse sources across the world, unlike other *Salmonella* serovars, did not exhibit rearrangements at these loci (21).

Strain 1656 was tested with all possible primer sets in a total of 49 reactions, and eight *rrn* operons were revealed—a remarkable difference from the standard seven operons which are normally present. It appears that *rrnC* is no longer present but there are two new hybrid *rrn* operons: *rrnC*/*A* and *rrnB*/*C*. This would indicate that the interribosomal I-*Ceu*I fragment E has been duplicated and one of the copies has translocated, i.e., recombined into the genome at *rrnC*, disrupting the operon and yielding two hybrids (Fig. 1B). Both the duplication and translocation events occurred as a result of recombination between the highly homologous *rrn* operons flanking each I-*Ceu*I fragment. However, it is unclear whether or not all eight *rrn* operons are functional in strain 1656.

Additional properties of strain 1656. Although this report focuses on genomic amplification, there were also a number of phenotypic differences between strain 1656 and the wild-type strain, as noted in Table 1. One major difference is that strain 1656, after starting in fresh Luria-Bertani broth from an overnight culture, had a longer lag phase as determined by a standard growth curve (data not shown). However, once the lag phase ended, the rate of growth in the exponential phase was essentially the same as that of the control strain. Upon reaching stationary phase, the optical density and colony counts were found to be tenfold lower than those of the control strain (data not shown).

DISCUSSION

It has been suggested that gene amplification may exist as a dynamic genomic state as one survival strategy that bacterial populations may use to adapt to changing environments. An excellent example is that presented by Romero and Palacios (27) whereby *Rhizobium* undergoes amplification during its invasion of legume roots in a complex nitrogen fixation process. There is some support for the concept that gene amplification gives cells a growth advantage under conditions of nutrient scarcity (28).

Additionally, it has been shown that *Salmonella* serovars that are specific for particular hosts and usually induce systemic typhoid-like diseases (such as serovars Typhi and Gallinarum),

have large-scale, stable genomic rearrangements at *rrn* operons (20). To date, no serovar Typhimurium isolated from nature has ever exhibited genomic rearrangements at *rrn* operons (21). Recently, archival mutator strains of serovar Typhimurium were shown to have undergone these sorts of rearrangements (19). However, this study represents the first report on nonmutator strains of Typhimurium with genomic rearrangements at *rrn* operons without direct selection. Because the only *Salmonella* strains with *rrn* rearrangements found in nature are host-specific pathogens, it is possible that the growth conditions that perpetuate these sorts of rearrangements may provide insight to the poorly understood differences between the host-specific and non-host-specific serovars of *Salmonella enterica*.

Chemostat selection experiments demonstrate that tandem chromosomal duplications allow improved bacterial growth under limiting concentrations of malate, arabinose, melibiose, or sorbitol. Such cells might be expected to lose the amplified region when ample nutrition returns. Lenski et al. (18) analyzed a population of *Escherichia coli* that has grown by 20,000 generations, via daily serial passage over the course of about 4 years, in the laboratory and noted numerous stable genomic insertion sequence transpositions and other types of chromosomal rearrangement.

The selection of cells with such amplified structures and subsequent rearrangements fits with evolutionary concepts. The microarray data support the view that deletions and amplifications spanning one or more genes are generally not the way that *Salmonella* adapted to long-term storage but that such deletions and amplifications do take place in a minority of strains. The homologous sequences among *rrn* operons would be ideal zones for amplifications, although there are other hot spots such as insertion sequence segments and loci containing long repeated sequences such as in *Haemophilus influenzae* with numerous repeats of bases (11). In fact, insertion sequences have been shown to be a hot spot for genomic rearrangements in *Salmonella*. Alokam et al. recently reported an inversion at IS*200* sites in serovar Typhi strain CT18 (1).

It may be expected that cells with amplified regions might be unstable with subsequent loss of the insertion. We have no evidence that this is the case with strain 1656. It was transferred through an estimated 1,000 generations before microarray and PCR analyses. Also, microarrays performed a full year apart with strain 1656 displayed the same histograms. When plated, colonies of strain 1656 were remarkably uniform in morphology. Recently we have observed rare smaller colonies, which we are now testing for change in any other phenotype.

To address the concern that mutations may have preexisted in the inoculated stabs decades ago, note that each auxotrophic culture was initially inoculated in quintuplicate from a single colony. Cells from "sibling" vials in the collection have been tested and exhibit genetic diversity to the same degree as those observed from cells in random vials. These observations support the view that the scored genetic changes occurred during storage and did not preexist.

The large amplification in the genome of strain 1656 in the region between *rrn* operons is particularly striking. Although this amplification was previously detected via PFGE by using a rare-cutting endonuclease, the precise location of the amplification and the subsequent translocation were not discovered until the present study (8). The only other reported amplification of this size in serovar Typhimurium is in the Ames TA104 strain with a 282-gene amplification that has an insertion sequence located at least on one end (25). For *Rhizobium*, a large natural amplification has been observed (12), and large duplications of 250 kb have also been found in *Mycobacterium smegmatis* (13). The precise mechanism by which these genomic amplifications and rearrangements occurred is not known. Homologous recombination at direct repeats results in duplications, deletions, and translocations, while recombination at homologous repeats in opposite orientations results in inversions (2, 22). In natural isolates of *Salmonella*, the homology required for these genomic changes has been observed to be as small as IS*200* elements (1) or as large as *rrn* operons (20). The precise join points for the duplications and deletions in this study have not yet been identified. Further analysis of these strains could identify the precise sequence at which the genomic changes occurred.

In summary, this collection of several thousand archival cultures may be a valuable resource for investigators with an interest in evolution, genome stability, or survival strategies of intracellular pathogens like *Salmonella enterica.*

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