

Research article

## Carbon and nitrogen substrate utilization by archival *Salmonella typhimurium* LT2 cells

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Published: 8 September 2002

Received: 9 May 2002

*BMC Evolutionary Biology* 2002, 2:14

Accepted: 8 September 2002

This article is available from: <http://www.biomedcentral.com/1471-2148/2/14>

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### Abstract

**Background:** A collection of over 20,000 *Salmonella typhimurium* LT2 mutants, sealed for four decades in agar stabs, is a unique resource for study of genetic and evolutionary changes. Previously, we reported extensive diversity among descendants including diversity in RpoS and catalase synthesis, diversity in genome size, protein content, and reversion from auxotrophy to prototrophy.

**Results:** Extensive and variable losses and a few gains of catabolic functions were observed by this standardized method. Thus, 95 catabolic reactions were scored in each of three plates in wells containing specific carbon and nitrogen substrates.

**Conclusion:** While the phenotype microarray did not reveal a distinct pattern of mutation among the archival isolates, the data did confirm that various isolates have used multiple strategies to survive in the archival environment. Data from the MacConkey plates verified the changes in carbohydrate metabolism observed in the Biolog™ system.

### Background

An extensive collection of *Salmonella typhimurium* auxotrophic mutants, accumulated by Miloslav Demerec and associates in the 1950 and 1960 decades was used primarily to develop intra- and inter-genic maps of the *S. typhimurium* LT2 chromosome [1,2]. Over 20,000 of these original sealed agar stab cultures (0.5 ml/agar stab vial), together with the original stockbooks, are now curated at the Cancer Research Center. The isolates were all descendants of a common parental strain [3] that was lyophilized around the same time that the original stab cultures were banked. The original mutants were very stable as indicated by low rates of reversion to prototrophy.

In previous reports, we noted extensive diversity among descendants of the parental strain, including diversity in RpoS and catalase synthesis [4], and diversity in genome size, protein content, and reversion from auxotrophy to prototrophy [5].

Funchain, *et al.* [6] reported that Biolog Phenotype Microarray™ (ES) plates test for loss of 350–400 gene functions. The ES Biolog plates that they used were designed to distinguish *E. coli* from *Salmonella* sp. Since their report, Biolog Phenotype Microarray™ PM1 and PM3 plates became available specifically for catabolism of carbon and nitrogen substrates, respectively. Use of these plates extends

**Table 1: Bacterial strains; summary of Biolog™ microassay results**

<b>Panel A. Archival <i>S. typhimurium</i> Strains</b>					
Our Lab #	Inoc. Date	Allele	# of Differences from Sequenced Strain		Total of C+N
			Carbon substrates	Nitrogen substrates	
<b>2004<sup>a</sup></b>	<b>4-01</b>	<b>WT</b>	<b>0</b>	<b>0</b>	<b>0</b>
1601	6-54	<i>his-144</i>	10	76	86
1915	8-59	<i>aro-50A</i>	5	73	78
1922	1-65	<i>thy-158</i>	12	62	74
1598	2-58	<i>his-141</i>	18	53	71
1702	8-64	<i>thy-315</i>	12	59	71
1695	8-64	<i>thy-272</i>	10	52	62
1717	8-62	<i>thy-320</i>	16	42	58
1703	8-64	<i>thy-314</i>	14	43	57
1597	2-58	<i>his-140</i>	9	48	57
1704	8-64	<i>thy-316</i>	8	45	53
1596	4-67	<i>his-2555</i>	10	38	48
1768 <sup>b</sup>	1-65	WT.	7	27	34
1927	8-57	<i>aro-20</i>	8	23	31
1699	8-64	<i>thy-314</i>	7	23	30
1935	8-59	<i>aro-39</i>	16	12	28
1690	8-64	<i>thy-271</i>	6	19	25
1962 <sup>c</sup>	9-65	B3 hfr	11	10	21
1923	4-58	<i>his-2526</i>	6	11	17
1693	8-64	<i>thy-274</i>	6	10	16
1687	8-64	<i>thy-273</i>	4	9	13
1707	8-62	<i>thy-320</i>	6	5	11
<b>Panel B. Non-archival reference strains</b>					
1602 <sup>d</sup>	10-98	$\chi$ 3000	8	22	30
1937 <sup>e</sup>	8-00	P.mult.	77	73	150
2000 <sup>f</sup>	3-00	WT	27	10	37

<sup>a</sup> Sequenced *S. typhimurium* LT2 strain<sup>b</sup> No longer auxotrophic <sup>c</sup> No longer auxotrophic; Hfr from *E. coli*<sup>d</sup>*S. typhimurium* LT2; from Cheryl Nickerson <sup>e</sup>*Pasteurella multocida*; American Type Culture Collection #4425-A. <sup>f</sup>*S. typhimurium* SL1344; Mass. Gen. Hospital

potential scoring of loss of non-essential genes to perhaps 15–20% of the entire genome.

To increase our knowledge of diversity among these archival strains, we scored for metabolic changes using Biolog Phenotype Microarray™ plates (ES, PM1 and PM3) followed by plating on MacConkey agar base supplemented with a variety of sugars. The ES and PM test panel provided a standardized microtiter method using 95 biochemical tests for each of the three 96-well plates to characterize and/or identify different metabolic profiles among microorganisms. Initially, 95 archival strains were tested on ES plates, the only Biolog™ plates available at that time (data

not shown). When PM1 and PM3 plates became available, we selected 21 strains that previously showed diversity from ES tests for further testing. Data from the MacConkey plates verified the changes in carbohydrate metabolism observed in the Biolog™ system.

## Results

As noted, experiments were started with ES plates designed as a diagnostic tool to distinguish between *E. coli* and *Salmonella* sp. When PM1 and PM3 plates became available, we chose 21 archival strains from the ES list to score for differences in catabolism of 190 different substrates with Biolog™ PM1 and PM3 plates. Nine additional

non-archival strains were tested including the sequenced *S. typhimurium* (our strain #2004) for comparison. Table 1 contains information on the background of the strains used for this study as well as other strains used for comparison and as controls. Table 1 also lists the number of differences between the sequenced reference strain (#2004) and archival strains. Thus, out of the 190 total carbon and nitrogen substrates, archival strain 1601 differed from 2004 in 86 (45%) of the tests. On the other hand, strain 1687, stored for approximately the same length of time, differed from 2004 in only 13 substrate utilizations (less than 7%).

In Panel B of Table 1, three non-archival strains are included. Note that *Pasteurella multocida* differed from strain 2004 in substrate utilization for 150 out of 190, an indicator of the validity of the Biolog™ system. The two non-archival *S. typhimurium* strains also displayed some differences from the sequenced strain, an indication that the Biolog™ PM1 and PM3 system might be useful in epidemiology and evolutionary studies.

Table 2 [see Additional file 1] lists the 86 carbon substrates (PM1 plate) catabolized by the sequenced strain 2004, and the number of archival strains (out of 21) that also catabolized these substrates. Thus, D-galacturonic acid was most easily dispensable (1/21), followed closely by adonitol (3/21) sucrose (5/21), alpha-D-lactose (6/21) and glyoxylic acid (7/21).

Table 3 lists the nine carbon substrates (PM1 plate) that were not metabolized by the sequenced 2004 strain. However, some archival strains did catabolize seven of these substrates, perhaps indicating alternative pathways (see DISCUSSION, below).

**Table 3: Archival strain utilization of carbon substrates on PM1 plates**

Substrate	# of archival strains (of 21) that catabolized the given substrate
Lactulose	0
D-Threonine	4
Glycolic Acid	1
D-Cellobiose	7
Acetoacetic Acid	2
D-Malic Acid	1
L-Galactonic Acid- $\gamma$ -Lactone	0
Phenylethylamine	2
2-Aminoethanol	5

Sequenced strain 2004 did not catabolize these 9 substrates. The number of archival strains that exhibited a positive phenotype for these substrates is shown in the second column.

Table 4 [see Additional file 2] lists the 84 nitrogen substrates (PM3 plate) catabolized by the sequenced strain 2004, and the number of archival strains (out of 21) that also catabolized these substrates. Thus, hydroxylamine and D,L-alpha-amino-caprylic acid were the most easily dispensable (2/21 for each), followed closely by D-amino-N-valeric acid (3/21) and 2-amino-N-valeric acid (5/21).

Table 5 lists the 11 nitrogen substrates (PM3 plate) that were not metabolized by the sequenced 2004 strain. However, several archival strains did catabolize these 11 substrates, perhaps indicating alternative pathways (see DISCUSSION, below).

**Table 5: Negative reactions of archival strains on PM3 plates**

Substrate	# of archival strains (of 21) that catabolized the given substrate
L-Glutamic Acid	8
D-Glutamic Acid	2
L-Homoserine	6
Hydroxylamine	2
Ethylenediamine	4
Putrescine	9
Agmatine	5
Guanine	2
Uric Acid	2
D,L- $\alpha$ Amino-Caprylic Acid	2
Ala-Asp	14

Sequenced strain 2004 did not catabolize these 11 substrates. The number of archival strains that exhibited a positive phenotype for these substrates is shown in the second column.

It is of interest that there are amino acids in both the carbon PM1 and the nitrogen PM3 plates. For example, the oxidation reaction for serine in PM1 identified carbon catabolism, while the ammonia moiety of serine was one enzyme, into a central pathway that can feed all other carbon pathways. Sometimes, but not always, the enzymes are the same, but induction of the enzymes under carbon or nitrogen starvation may be different [[7,8] and Bochner, personal communication].

## Discussion

Bochner, *et al.*[7] emphasize that the PM plates may be used to analyze the linkage between genotype and phenotype. They point out that scoring of catabolic loss is identified in the PM3 tests. Amino acids can be used as nitrogen sources since in many cases they only need a single enzyme such as a transaminase or deaminase to liberate the ammonia. Some amino acids can also be used as a carbon source when their carbon skeletons are converted,

usually by more than one substrate, and can reveal the loss of shared transport functions or shared catabolic enzymes. The use of PM plates have several major benefits to the analysis of our strains: 1) color change is easy to monitor and quantitate; 2) color change is very sensitive and highly reproducible; and 3) cellular respiration can occur independent of cell growth and, in some cases, can measure phenotypes that do not lead to growth [7]. Other researchers have used the Biolog™ System to explore other phenotypic changes in various mutated strains of bacteria [8].

In these archival cultures, it is assumed that the initial nutritional components were gradually depleted; however, some new molecules became available upon decomposition of dead cells. Mutant offspring that were best able to utilize the sparse nutritional residues would survive. Our long-term focus has been to characterize genetic differences between survivors and the putative parent, the sequenced *S. typhimurium* LT2 strain. A goal is to develop gradients of preference from "most-to-least dispensable" gene functions. As may be seen in Tables 2 and 3, we observed a hierarchy of substrate utilization losses among strains.

The additional observation that some archival strains gained metabolic capabilities is consistent with the reports that environmental pressures may induce changes in metabolic pathways [7,9–14]. Entire metabolic pathways could be deactivated by the loss of a critical enzyme [7]. Bochner, *et al.* [7] noted that when a mutation is inserted to inactivate a xylose catabolic gene (*xylA*) metabolism of maltose and maltotriose were also lost in addition to xylose. Some of the primary energy sources in these archival cultures were likely to be exhausted, and thus cells might be expected to utilize secondary pathways, as noted by Groat, *et al.* [9] and Wright [10]. DNA from dead cells might serve as a source of energy for bacterial cells. Finkel and Kolter [15] observed that cells that were able to utilize DNA have a significant gain in fitness over competing cells that could not "eat" DNA. Five wells in the PM1 plate and seven in the PM3 plate contained nucleic acid components and mutants were able to utilize these both as carbon and nitrogen sources.

Koch [16] emphasized that certain genes could be eliminated without losing colony-forming ability. Among "excess baggage" genes, Koch stated that DNA fragments might include duplicated genes or fragments, fragments of ancestral genes that are no longer needed as the species evolved, and temperate viruses and insertion sequences. Although constitutive pathways might be needed to utilize the meager remaining nutrients, there would be less need for induction processes. Ochman and associates [17–19]. Papadopoulos, *et al.* [20] and Arber [21] have

presented interesting evidence of such genomic losses. Mutation under nutrient deprivation has been of interest from the dawn of bacterial genetics.

Decades ago, Novick and Atwood [22,23] both reported that starvation serves as a selective condition for mutants with survival capabilities over the parental strain. In examining whether there was any priority in discarding of excess baggage in the population of cells in these vials, certain features would be obvious without any sophisticated analysis. An indication of this in the greater than expected reversion from auxotrophy to prototrophy and in the PFGE data [5].

## Conclusions

It is premature to build a model as to precise genetic changes during decades of storage in this restricted environment. However, our results support the view that: 1) Gene functions are lost or rearranged in almost all (if not all) of the archival strains tested. 2) There may be a hierarchy from "most-dispensable-to-least dispensable" substrate utilization for both carbon and nitrogen molecules. (3) Since some food components would be greatly diminished under starvation conditions, selected mutants in populations may discover secondary pathways.

## Methods

Upon opening of sealed vials, 0.5 ml of buffered saline was added. The agar-saline mix was thoroughly mashed. The mix was allowed to remain at room temperature for 30 minutes so that the large particles would settle to the bottom of the tube and additional cells might be leached out of the agar. Samples were divided into several containers, and subsequently handled in a manner depending on specific questions to be addressed. Aliquots were held aside for storage at -80°C in 50% glycerol.

## Phenotype microarrays

Cultures were taken from storage at minus 80°C and incubated in Luria-Bertani Broth at 37°C with aeration overnight. After incubation, cultures were centrifuged for 5 minutes at 3,000 g. Following centrifugation, the supernatant was decanted and inoculating fluid from Biolog™ was added to adjust the turbidity of the cell suspension to an absorbency of 0.105. For analysis of Biolog™ PM3 plates 150 µl ferric citrate was also added to each tube of inoculating fluid. All necessary nutrients and biochemicals were pre-filled and dried into 95 wells of the plates plus a water control. Tetrazolium violet was used as a redox dye to colorimetrically indicate the utilization of the carbon and nitrogen sources.

The microtiter plates were pre-warmed at 37°C before each well was inoculated with 100 µl of cell suspension. In wells containing a substrate that is oxidizable, a burst

of respiration occurred and the cells reduced the tetrazolium dye forming a purple color. Other wells remained colorless (as an indicator of loss of function in a catabolic step), as did the reference well containing water. The PM1 and PM3 plates were incubated at 37°C for 24 and 36–48 hours respectively. Following incubation, the metabolism of the various carbon and nitrogen sources was measured spectrophotometrically at 570 nm, using a microtiter plate reader, and visually by scoring purple reactions in individual wells as a colorless negative control. The sequenced reference strain (#2004) was included with each experimental group as a control. The biochemical reactions resulting in tetrazolium color changes have been described by Bochner *et al.*[7].

#### Archival strains

The archival cultures were originally stocked beginning in the late 1950s for the purpose of intra- and intergenetic mapping. Each strain was auxotrophic for an amino acid, vitamin, or nucleotide. The isolates were stored in quintuplicate in small glass vials filled with nutrient agar and then sealed with paraffin. The collection has been stored at room temperature for over 40 years. While many of the tubes have dried out, all tubes in which agar was not dried out yielded an average of  $10^3$ – $10^5$  colony-forming units. Details of the strains used can be found in Table 1 with additional detailed information at [http://www.cancerresearchcenter.org/table1.html].

#### Reference strain

Our reference strain (#2004) is assumed to be the parent of the LT2 archival strains. It was lyophilized at about the same time that the archival collection was being stored in agar stabs. The same strain was used for sequencing of the *S. typhimurium* genome completed at Washington University sequencing center. A paper detailing the sequence information [3] is now available at [http://genome.wustl.edu]. Also, a detailed tabulation by Gutnick, *et al.*[24] in 1969 of compounds of carbon and nitrogen for *S. typhimurium* LT2 gave us an additional basis of comparison of our results with a strain studied decades ago.

#### MacConkey Agar

Single colony suspensions were added to a MacConkey Agar Base (Difco, Detroit, MI.) supplemented with 15 different sugars. One-percent sugar (w/v) was added to each sector of a four-quadrant plate. A sector containing XLD (a selective media for *Salmonella* comprised of xylose, lysine, sodium desoxycholate, sodium thiosulfate, and ferric ammonium citrate) (Difco, Detroit, MI) was also included as an additional phenotypic test. The results of this assay provide information on as many as 50 different genes and their associated activity. A complete listing of the sub-

strates in all of the Biolog™ plates may be found at [http://www.Biolog.com].

#### Authors' contributions

Author 1 (BST) carried out the phenotype microarray studies, participated in the confirmation tests, compiled data for and edited the manuscript. Author 2 (KKE) carried out the biochemical confirmation testing, directed the compilation and analysis of the phenotypic data, and drafted the manuscript. Author 3 (AE) conceived the study and participated in its design, coordination and reporting.

#### Additional material

##### Additional file 1

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##### Additional file 2

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#### Acknowledgments

The authors wish to acknowledge the financial support of the Cancer Research Center Board of Directors.

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