RESEARCH LETTER

Role of a short tandem leucine/arginine repeat in strong mutator phenotype acquisition in a clinical isolate of Salmonella Typhimurium

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Salmonella; mutator; mismatch repair; mutL; short tandem repeat.

Abstract
In this prospective study, a strong mutator strain of Salmonella Typhimurium was isolated from a collection of 130 human clinical strains of Salmonella. Sequence analysis of the mutS, mutL, and mutH genes, which encode three proteins that are essential for initiation of methyl-directed DNA mismatch repair, revealed insertion of a short tandem repeat (STR) of leucine/alanine in the histidine kinase-like ATPase domain of MutL. The role of this STR in the acquisition of the strong mutator phenotype was confirmed by the construction of an isogenic mutant (6bpinsmutL) from a normomutator strain of Salmonella Heidelberg. This result adds to the sparse body of knowledge about strong mutators and highlights the role of this STR as a hotspot for the acquisition of a strong mutator phenotype in Salmonella.

Introduction
As a leading etiological agent in bacterial foodborne diseases and the causative agent of typhoid and paratyphoid fevers (serotypes Typhi and Paratyphi A and B), Salmonella bacteria can thrive in stressful conditions both in host organisms and in the environment. Such conditions may favor mutations that help these bacteria adapt to a hostile environment (Galhardo et al., 2007). The prevalence of strong mutators, which are characterized by an increased frequency of spontaneous mutations, ranges from about 1% among pathogenic strains of Escherichia coli (Baquero et al., 2004) to more than 30% among Pseudomonas aeruginosa stains isolated from cystic fibrosis patients (Oliver et al., 2000). The role of the strong mutator phenotype in pathogenic bacteria has been discussed at great length (Jolivet-Gougeon et al., 2011), but the link between this phenotype and virulence is not yet well understood. However, a strong mutator phenotype is expected to drive adaptation to a hostile environment (Taddei et al., 1997). Strong mutators are detected easily by enumeration of antibiotic-resistant mutants on culture media containing rifampicin, fosfomycin, nalidix acid, streptomycin, or spectinomycin (LeClerc et al., 1996; Matic et al., 1997). Polymorphisms in rifampicin resistance genes have been studied by Baquero et al. (2004), who arbitrarily defined four categories of E. coli strains according to their mutation frequencies (f) as follows: hypomutator (f ≤ 8 × 10−9), normomutator (8 × 10−9 < f < 4 × 10−8), weak mutator (4 × 10−8 ≤ f < 4 × 10−7), and strong mutator (f ≥ 4 × 10−7). In most cases, the mutator phenotype is due to a defective methyl mismatch repair (MMR) system (LeClerc et al., 1996), which plays a key role in the correction of base–base mismatches and insertion/deletion mispairs that appear during DNA replication. MutS, MutL, and MutH are...
three bacterial proteins that are essential for initiation of methyl-directed DNA mismatch repair (Li, 2008).

The objectives of this study were to determine the prevalence of mutators among human clinical isolates of Salmonella by prospective screening and to characterize the detected strong mutators by sequencing the MMR genes to find short tandem repeats (STRs).

Materials and methods

Bacterial strains and plasmids

This study included all strains of Salmonella (n = 130) collected from clinical samples between the 1st of March 2009 and the 30th of April 2010 in seven French hospital laboratories. The hospitals were located in Angers, Brest, Lorient, Quimper, Rennes, Saint-Brieuc, and Vannes. In cases of outbreaks, only the first isolated strain was included. The great majority of strains were isolated from stools (n = 119). The remaining strains were isolated from blood (n = 7), intestinal biopsies (n = 2), urine (n = 1), and hematoma (n = 1) (Table 1).

Mutation frequency

Rifampicin and fosfomycin resistance mutation frequencies were determined as described previously (Le Clerc et al., 1996; Denamur et al., 2002). Briefly, a single colony of the bacterial strain was suspended in 10 mL LB broth (AES Laboratory) and incubated at 37 °C for 24 h. One hundred microliters of this culture were spread onto LB agar plates with and without rifampicin (Sigma Aldrich) at 100 µg mL⁻¹ or fosfomycin (Sigma Aldrich) at 30 µg mL⁻¹. The mutation frequencies were reported as the ratio of the number of antibiotic-resistant colony-forming units per ml (CFU mL⁻¹) to the number of CFU mL⁻¹ on LB agar plates lacking antibiotics that were plated in parallel. For each strain, mutation frequencies were determined in triplicate, and the mean of the values was calculated for each serotype (Fig. 1).

DNA sequencing

Sequences of the mutS, mutL, and mutH genes were determined on both strands by Sanger’s method (Sanger et al., 1977) using the Applied Biosystems model 3130 DNA sequencer and Dye Primer kits (ABI, Foster City, CA). Sequences were analyzed using CHROMASPRO (v.1.5) software (http://www.techneley.com.au) and converted to amino acid sequences using in silico simulation (http://insilico.ehu.es). The primers used in this study are listed in Table 2.

Construction of 6bpinsmutL by allelic exchange

We generated 6bpinsmutL, the isogenic mutant of the normomutator Salmonella Heidelberg wt (Le Gall et al., 2009), by allelic exchange with the mutL allele of the strong mutator STM HS20 detected in this study as described previously (Philippe et al., 2004). The cloning steps, which used the primers described in Table 2, were performed in SM10λpir strains (LMBP 3889, BCCM/ LMBP plasmid collections, Gent, Belgium) to allow replication of the pPDS132 plasmid containing the mutL allele of STM HS20.

Results

Prevalence of mutators

Salmonella Typhimurium was the most frequent serotype (n = 66) among the 130 isolated strains. It was followed by serotype Enteritidis (n = 18) and the monophasic variant 4,5,12:i:- (n = 15) (Echeita et al., 1999). This is similar to the nationwide serotype distribution for the same period (Weill & Le Hello, 2009).

Mutation frequencies were first determined using rifampicin-containing media as described previously (Le Clerc et al., 1996; Le Gall et al., 2009) (Table 1). Polymorphisms in rifampicin resistance genes have been studied by Baquero et al. (2004), who arbitrarily defined four categories of E. coli strains according to their mutation frequencies (f) as follows: hypomutator (f ≤ 8 × 10⁻⁹), normomutator (8 × 10⁻⁹ < f < 4 × 10⁻⁸), weak mutator (4 × 10⁻⁸ < f < 4 × 10⁻⁷), and strong mutator (f ≥ 4 × 10⁻⁷). Salmonella strains were classified using the system developed for E. coli by Baquero et al. (2004), as follows: 33 (25.6%) were hypomutators, 75 (58.1%) were normomutators, 20 (15.5%) were weak mutators, and 1 (0.77%) was a strong mutator. The latter strain, which was serotype Typhimurium, was called STM HS20 (rifampicin resistance mutation frequency, f = 4.8 × 10⁻⁶ ± 4.9 × 10⁻⁶) and was confirmed as a strong mutator by measuring the fosfomycin resistance mutation frequencies (f = 1.8 × 10⁻⁴ ± 8.5 × 10⁻⁵).

Molecular characterization of STM HS20

Alignment analysis of the mutL allele of STM HS20 with S. Typhimurium LT2 (NC_003197) and the normomutator Salmonella serotype Heidelberg wt (Le Gall et al., 2009) using CLUSTALW (http://clustalw.genome.jp) revealed the
Table 1. Serotypical distribution and mutation frequencies of the Salmonella strains used in this study

<table>
<thead>
<tr>
<th>Identification</th>
<th>Number of isolates (n)</th>
<th>Mean rifampicin resistance mutation frequencies* (±SD)</th>
<th>Strain characteristics and references</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clinical strains</strong></td>
<td></td>
<td></td>
<td>Clinical strains isolated from human stools (this work)</td>
</tr>
<tr>
<td>Salmonella enterica ssp. enterica</td>
<td>130</td>
<td>1.9 × 10⁻⁸ ± 1.7 × 10⁻⁸</td>
<td></td>
</tr>
<tr>
<td>Typhimurium</td>
<td>65</td>
<td>4.8 × 10⁻⁶ ± 4.9 × 10⁻⁶</td>
<td></td>
</tr>
<tr>
<td>Typhimurium HS20</td>
<td>1</td>
<td>2.3 × 10⁻⁸ ± 1.4 × 10⁻⁸</td>
<td></td>
</tr>
<tr>
<td>Enteritidis</td>
<td>18</td>
<td>1.3 × 10⁻⁸ ± 7.2 × 10⁻⁹</td>
<td></td>
</tr>
<tr>
<td>4,5,12:i:-</td>
<td>15</td>
<td>5.0 × 10⁻⁸ ± 3.4 × 10⁻⁸</td>
<td></td>
</tr>
<tr>
<td>Augustenborg</td>
<td>2</td>
<td>1.4 × 10⁻⁸ ± 7.1 × 10⁻⁹</td>
<td></td>
</tr>
<tr>
<td>Chester</td>
<td>2</td>
<td>8.3 × 10⁻⁸ ± 5.1 × 10⁻⁸</td>
<td></td>
</tr>
<tr>
<td>Concord</td>
<td>2</td>
<td>4.3 × 10⁻⁹ ± 1.8 × 10⁻⁹</td>
<td></td>
</tr>
<tr>
<td>Hadar</td>
<td>2</td>
<td>9.6 × 10⁻⁸ ± 6.2 × 10⁻⁸</td>
<td></td>
</tr>
<tr>
<td>Infantis</td>
<td>2</td>
<td>3.6 × 10⁻⁸ ± 2.7 × 10⁻⁸</td>
<td></td>
</tr>
<tr>
<td>Kottbus</td>
<td>2</td>
<td>1.2 × 10⁻⁸ ± 7.8 × 10⁻⁹</td>
<td></td>
</tr>
<tr>
<td>Paratyphi A</td>
<td>2</td>
<td>2.5 × 10⁻⁸ ± 1.1 × 10⁻⁸</td>
<td></td>
</tr>
<tr>
<td>Ajibio</td>
<td>1</td>
<td>5.0 × 10⁻⁸ ± 3.7 × 10⁻⁸</td>
<td>Rifampicin resistant strain¹</td>
</tr>
<tr>
<td>Braenderup</td>
<td>1</td>
<td>7.2 × 10⁻⁸ ± 3.8 × 10⁻⁸</td>
<td></td>
</tr>
<tr>
<td>Brandenburg</td>
<td>1</td>
<td>8.1 × 10⁻⁹ ± 7.2 × 10⁻⁹</td>
<td></td>
</tr>
<tr>
<td>Redeney</td>
<td>1</td>
<td>3.6 × 10⁻⁸ ± 9.1 × 10⁻⁹</td>
<td></td>
</tr>
<tr>
<td>Dublin</td>
<td>1</td>
<td>1.0 × 10⁻⁸ ± 6.6 × 10⁻⁸</td>
<td></td>
</tr>
<tr>
<td>Havana</td>
<td>1</td>
<td>2.3 × 10⁻⁸ ± 7.5 × 10⁻⁹</td>
<td></td>
</tr>
<tr>
<td>Heidelberg</td>
<td>1</td>
<td>6.3 × 10⁻⁹ ± 7.0 × 10⁻⁹</td>
<td></td>
</tr>
<tr>
<td>Kedougou</td>
<td>1</td>
<td>2.3 × 10⁻⁸ ± 1.5 × 10⁻⁸</td>
<td></td>
</tr>
<tr>
<td>Manhattan</td>
<td>1</td>
<td>6.0 × 10⁻⁹ ± 1.0 × 10⁻⁹</td>
<td></td>
</tr>
<tr>
<td>Montevideo</td>
<td>1</td>
<td>1.2 × 10⁻⁸ ± 8.6 × 10⁻⁹</td>
<td></td>
</tr>
<tr>
<td>Muenster</td>
<td>1</td>
<td>1.2 × 10⁻⁸ ± 8.5 × 10⁻⁹</td>
<td></td>
</tr>
<tr>
<td>Napoli</td>
<td>1</td>
<td>5.6 × 10⁻⁸ ± 1.0 × 10⁻⁹</td>
<td></td>
</tr>
<tr>
<td>Nima</td>
<td>1</td>
<td>3.1 × 10⁻⁸ ± 8.2 × 10⁻⁹</td>
<td></td>
</tr>
<tr>
<td>Ttheidington</td>
<td>1</td>
<td>1.9 × 10⁻⁸ ± 1.2 × 10⁻⁸</td>
<td></td>
</tr>
<tr>
<td>Typhi</td>
<td>1</td>
<td>1.6 × 10⁻⁸ ± 1.1 × 10⁻⁸</td>
<td>Gong et al. (2007)</td>
</tr>
<tr>
<td>Virchow</td>
<td>1</td>
<td>3.0 × 10⁻⁸ ± 3.4 × 10⁻⁸</td>
<td>Jolivet-Gougeon et al. (2011)</td>
</tr>
<tr>
<td>Salmonella enterica subsp. arizonae</td>
<td>1</td>
<td>9.2 × 10⁻⁶ ± 1.4 × 10⁻⁶</td>
<td>wt derivative obtained by allelic exchange using pDS132 containing the MutL allele version of STM HS20 (this work)</td>
</tr>
<tr>
<td><strong>Control strains and constructs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salmonella Typhimurium LT2</td>
<td></td>
<td>1.6 × 10⁻⁸ ± 1.1 × 10⁻⁸</td>
<td></td>
</tr>
<tr>
<td>Salmonella Heidelberg wt</td>
<td></td>
<td>3.0 × 10⁻⁸ ± 3.4 × 10⁻⁸</td>
<td></td>
</tr>
<tr>
<td>Salmonella Heidelberg 6bpinsmutL</td>
<td></td>
<td>9.2 × 10⁻⁶ ± 1.4 × 10⁻⁶</td>
<td></td>
</tr>
</tbody>
</table>

*Mutation frequencies were defined as the ratio of the number of rifampicin-resistant CFU mL⁻¹ to the number of CFU mL⁻¹ on LB agar plates lacking antibiotics that were plated in parallel.

¹This strain was classified as normomutator because of the mutation frequency in response to fosfomycin.

insertion of six nucleotides (CTGGCG) at position 214. This resulted in the insertion of a leucine (L) residue and an alanine (A) residue at amino acid sequence positions 72 and 73, respectively. Analysis of the sequence revealed that the inserted nucleotide pattern (CTGGCG) corresponded to a STR that was repeated three times in the mutL allele of normomutator strains of Salmonella. Analysis of the three-dimensional structure of E. coli MutL, which was reported by Ban et al. (1999) and added to the Molecular Modeling Database by Wang et al. (2007), revealed that this LA insertion is localized in the histidine kinase-like ATPase domain of MutL. The ATPase activity of MutL, which is required for mismatch repair (Spaminato & Modrich, 2000), may be altered in STM HS20.

The role of the CTGGCG insertion in the mutator phenotype was confirmed by the strong mutator phenotype of 6bpinsmutL (Table 1), which is the isogenic mutant of the normomutator Salmonella serotype Heidelberg wt (Le Gall et al., 2009).

**Discussion**

In previous retrospective studies, strong mutators among Salmonella strains have been observed with variable
frequencies: 3.6% (LeClerc et al., 1996), 0.7% (Baquero et al., 2004), or 0.77% (Le Gall et al., 2009), but far lower than 36%, which is the frequency of strong mutators among P. aeruginosa strains isolated from cystic fibrosis patients (Oliver et al., 2000). Our work is a prospective study, while previous ones were retrospective and therefore susceptible to bias because they were conducted after the strains had been stored for a long time. Importantly, mutational events can occur during storage (Ferenci et al., 2009) or prolonged starvation, and such events can modify genes, including those belonging to the MMR system (Gong et al., 2007).

In this work, we demonstrated that insertion of the STR CTGGCG in mutL leads to a strong mutator phenotype in Salmonella. Deletion of this STR had already been described in an archival strong mutator strain derived from S. Typhimurium LT7 that was stored at room temperature in agar stabs for about four decades (Gong et al., 2007). This STR is also present in the nucleotide sequence of mutL in E. coli, and there are two spontaneously originating strong mutators that were characterized previously that showed a deletion or an insertion of this STR (Shaver & Snigowski, 2003). The detection of deletions or insertions of the same STR in mutL in three independent experiments confirmed its previously suspected role as a hotspot involved in the acquisition of a strong mutator phenotype in Salmonella and E. coli (Rocha et al., 2002). Chen et al. (2010) found deletions in a region that forms the lid of the ATP-binding pocket, with a LALALA missing in MutL, playing a role in modulating bacterial mutability in Salmonella constructed strains. Modifications of the number of CTGGCG STRs in mutL may drive

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer name</th>
<th>Primer sequence, 5′–3′</th>
</tr>
</thead>
<tbody>
<tr>
<td>mutS</td>
<td>mutS long 1</td>
<td>GAGCATGTCGACTAATTGC</td>
</tr>
<tr>
<td></td>
<td>mutS long 2</td>
<td>CTTTATACGGGCTGTTGCA</td>
</tr>
<tr>
<td></td>
<td>mutS3</td>
<td>GGCAGGCTCTGGGAAATTTT</td>
</tr>
<tr>
<td></td>
<td>mutS4</td>
<td>AAGTGCGTCGACTAATGCG</td>
</tr>
<tr>
<td></td>
<td>mutS6</td>
<td>CGACAGTCAGCCTGAAAAAA</td>
</tr>
<tr>
<td></td>
<td>mutS8</td>
<td>GTAGCCGAAAATGCTTAAC</td>
</tr>
<tr>
<td></td>
<td>mutS10</td>
<td>GAATTAACCTGGCAGGACAT</td>
</tr>
<tr>
<td></td>
<td>mutS12</td>
<td>CGCCGTGCGCCGTTGGA</td>
</tr>
<tr>
<td>mutL</td>
<td>mutL1</td>
<td>TATATACGGGGGTTGGCTGA</td>
</tr>
<tr>
<td></td>
<td>mutL2</td>
<td>TAAATTGGGGCGGAATCAAC</td>
</tr>
<tr>
<td></td>
<td>mutL3</td>
<td>GCTTCCTGCAAGCAGTCTG</td>
</tr>
<tr>
<td>mutH</td>
<td>mutH5F</td>
<td>CCCACCTGCTATACGTG</td>
</tr>
<tr>
<td></td>
<td>mutH5R</td>
<td>GGCGAATACGGTCTAAG</td>
</tr>
</tbody>
</table>

Primers used to clone the mutL allele of STM HS20 in the pDS132 plasmid

<table>
<thead>
<tr>
<th>Primer sequence, 5′–3′</th>
</tr>
</thead>
<tbody>
<tr>
<td>mutL-Sacl</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>mutL-Sphi</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>
spontaneous conversions between the strong mutator and normomutator phenotypes, as has been described recently for MMR-converting prophages that are integrated into mutL in *Streptococcus pyogenes* (Scott et al., 2008). Still, strong mutator phenotypes caused by mutL mutations are very frequent in *P. aeruginosa*, at least in the cystic fibrosis setting (Mena et al., 2008). It is interesting to note that there is no such hotspot STR for the acquisition of a strong mutator phenotype in *P. aeruginosa* MMR genes (Feliziani et al., 2010). As expected from computer simulations of clonal populations adapting to a new environment (Taddei et al., 1997), CTGGCG insertions or deletions may hitchhike on a strong mutator genotype, generate favorable mutations, and drive adaptive radiation (Rainey & Travisano, 1998). The conditions that lead to conversions between mutator and normomutator phenotypes are not yet well understood. There are clear examples in nature such as antibiotic resistance (Maciá et al., 2005) or adaptation in chronic infections (Mena et al., 2008). In the strong mutator ST22 strain detected in this work, the ATPase activity of MutL, which is required for mismatch repair (Spampinato & Modrich, 2000), may be altered by the insertion of LA in the ATPase domain of the protein. This observation suggests that a possible link between the acquisition of a strong mutator phenotype and ATP consumption may exist. The conditions that lead to conversions between strong mutator and normomutator phenotypes are not yet well understood.

Thus, the study of strong mutator strains, especially clinical ones as such as described in this work, may help expand our knowledge and provide clinically useful information given that there is a high prevalence of strong mutators among strains, not only observed in constructed mutants, but also in pathogenic clinical specimens.

Acknowledgements

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References


