

Further supplemental information and code are available on GitHub at www.github.com/dlato/Spatial_Patterns_of_Substitutions.

Sequences

Bacteria Strain/Species	Accession Number	Date Accessed
<i>Escherichia coli</i>		
<i>E. coli</i> 0104H4	CP003289	September 29, 2016
<i>E. coli</i> 0157H7	BA000007	September 29, 2016
<i>E. coli</i> 083H1	CP001855	September 29, 2016
<i>E. coli</i> IAI39	CU928164	September 26, 2016
<i>E. coli</i> K12 *	U00096	September 26, 2016
<i>E. coli</i> UMN026	CU928163	September 26, 2016
Outgroup: <i>Salmonella enterica</i> LT2	AE006468	September 29, 2016
<i>Bacillus subtilis</i>		
<i>B. subtilis</i> 168 *	NC_000964	November 10, 2016
<i>B. subtilis</i> BS38	NZ_CP017314	November 11, 2016
<i>B. subtilis</i> BSn5	NC_014976	November 11, 2016
<i>B. subtilis</i> PY79	NC_022898	November 11, 2016
<i>B. subtilis</i> QB928	NC_018520	November 11, 2016
<i>B. subtilis</i> RONN1	NC_017195	November 11, 2016
<i>B. subtilis</i> W23	NC_014479	November 11, 2016
Outgroup: <i>Listeria monocytogenes</i> EDGe	NC_003210	November 11, 2016
<i>Streptomyces</i>		
<i>S. lividans</i> TK24	NZ_GG657756	April 28, 2017
<i>S. lividans</i> 1362	NZ_CM001889	April 28, 2017
<i>S. coelicolor</i> A3 *	AL645882	November 30, 2016
Outgroup: <i>Mycobacterium tuberculosis</i> H37Rv	NC_000962	November 30, 2016
<i>S. meliloti</i> Chromosome		
<i>S. meliloti</i> 2011	NC_020528	April 24, 2017
<i>S. meliloti</i> 1021 *	NC_003047	June 3, 2014
<i>S. meliloti</i> AK83	NC_015590	June 3, 2014
<i>S. meliloti</i> BL225C	NC_017322	June 3, 2014
<i>S. meliloti</i> SM11	NC_017325	June 3, 2014
<i>S. meliloti</i> RMO17	NC_CP009144	April 24, 2017
Outgroup: <i>Agrobacterium tumefaciens</i> C58 chromosome	AE007869	December 19, 2015
<i>S. meliloti</i> pSymA		
<i>S. meliloti</i> 2011	NC_020527	April 24, 2017
<i>S. meliloti</i> 1021 *	NC_003037	June 3, 2014
<i>S. meliloti</i> AK83	NC_015591	June 3, 2014
<i>S. meliloti</i> BL225C	NC_017324	June 3, 2014
<i>S. meliloti</i> SM11	NC_017327	June 3, 2014
<i>S. meliloti</i> RMO17	NC_CP009145	April 24, 2017
Outgroup: <i>Agrobacterium tumefaciens</i> C58 plasmid	AE007872	Jan 11, 2016
<i>S. meliloti</i> pSymB		
<i>S. meliloti</i> 2011	NC_020560	April 24, 2017
<i>S. meliloti</i> 1021 *	NC_003078	June 3, 2014
<i>S. meliloti</i> AK83	NC_015596	June 3, 2014
<i>S. meliloti</i> BL225C	NC_017323	June 3, 2014
<i>S. meliloti</i> SM11	NC_017326	June 3, 2014
<i>S. meliloti</i> RMO17	NC_CP009146	April 24, 2017
Outgroup: <i>Agrobacterium tumefaciens</i> C58 chromid	NC_003063	May 1, 2017

Table S1: Strains and species used for each replicon analysis. Accession numbers, date accessed, and outgroups for each replicon are provided. An asterisk (*) indicates the strain that was used as the representative strain.

Constraints to Number of Sequence Chosen

Computational time constraints and the nature of the data were limiting factors for the number of strains that were chosen for each bacterial species. **progressiveMauve** is a multiple sequence alignment program which is useful for accounting for local and large scale genomic rearrangements. Some of the bacterial strains are very similar and therefore there was no issue finding a sufficient number of locally co-linear blocks (LCBs) without having the genomes broken into an overwhelming number of blocks. *E. coli*, *Bacillus subtilis*, and *S. meliloti* were among the bacteria where this was the case. However, the *Streptomyces* strains were slightly too distantly related so when we tried to use a comparable number of strains to the other bacteria (six genomes), **progressiveMauve** split the genomes into 521 LCBs (Supplementary Figure S1). These blocks were therefore very small in length and resulted in many blocks that were comparing sequences with poor homology. Consequently, we had to reduce the number of genomes used for the *Streptomyces* analysis and after many iterations of genome combinations, we settled on three *Streptomyces* genomes with a total of 6 LCBs (Supplementary Figure S2). This allowed for the correct comparison of homologous sequences, while also accounting for recombination.

The computational time required to run **progressiveMauve** was an additional constraint that needed to be considered. **progressiveMauve** can align multiple whole genomes and identify regions that have been rearranged within the taxa provided. This process happens in relatively quick computational time, however, like most other programs, the addition of more data increased the amount of time required to complete the process. We ran multiple instances of **progressiveMauve** with varying numbers of *E. coli* genomes (Supplementary Figure S3). These data points were connected using a locally estimated scatterplot smoothing method and confidence intervals (Supplementary Figure S3). From this data, we can see that increasing the number of genomes exponentially increases the run time of **progressiveMauve**. It becomes impractical to align more than 27 genomes with **progressiveMauve**, as anything over that would take more than 24h to run. This information combined with **progressiveMauve**'s inability to pair of homologous sequences in LCBs of distantly related taxa, has limited the total number of genomes we can use per taxa to a maximum of 7. This provides the most accurate data and the most reasonable analysis duration.

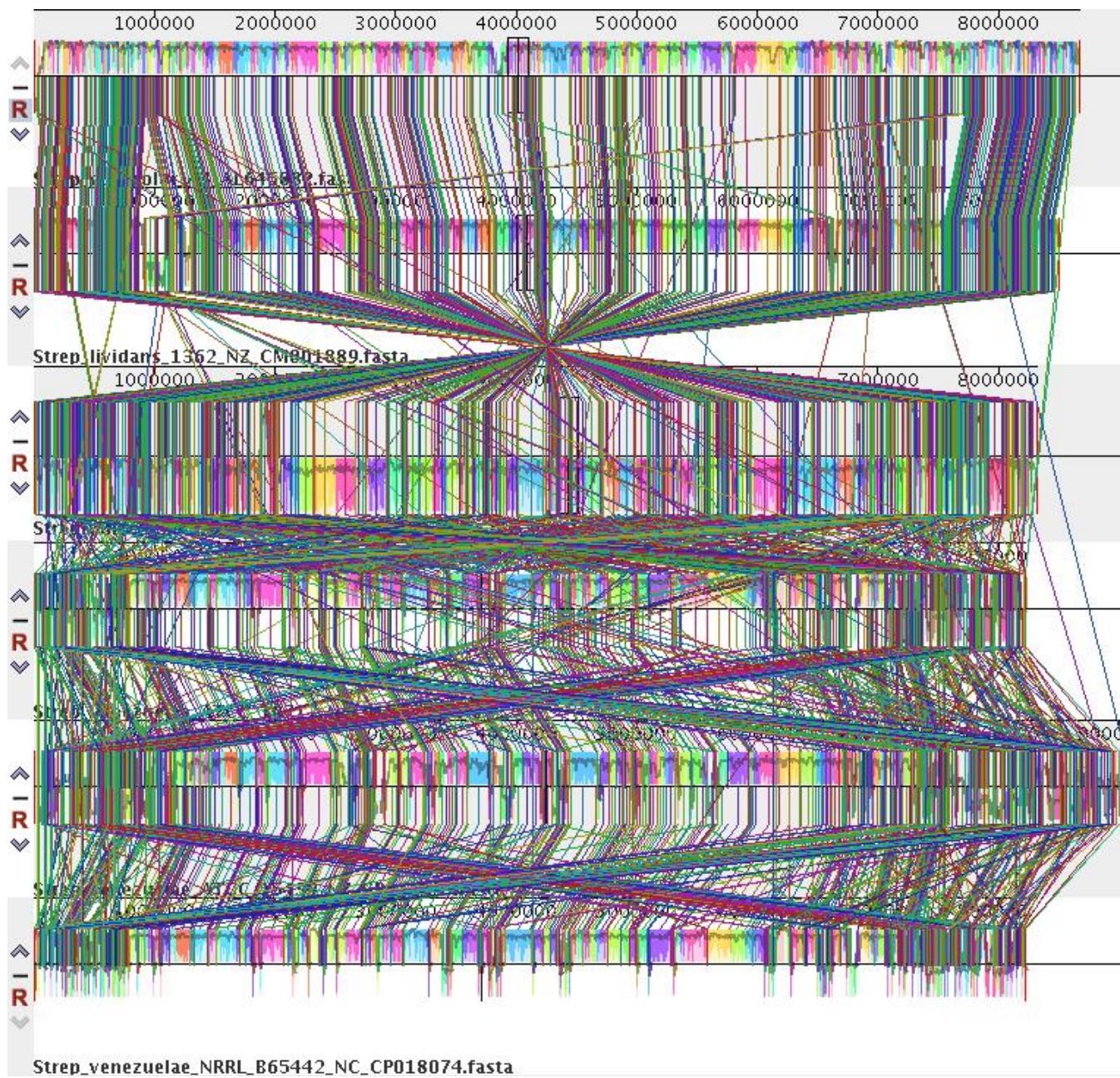


Figure S1: Visualization of the progressiveMauve alignment of 6 *Streptomyces* genomes (from top to bottom): *S. coelicolor* AL645882, *S. lividans* NZ_CM001889, *S. lividans* NZ_GG657756, *S. venezuelae* NC_018750, *S. venezuelae* NZ_CP013129, and *S. venezuelae* NC_CP018074. Each coloured block represents a different locally co-linear block (LCB). Coloured lines connect LCBs that are similar between taxa. The black lines underneath each LCB represent the whole genome sequence of each of the *Streptomyces* taxa. Each LCB can be treated as a rearrangement, there have therefore been 521 rearrangements between these *Streptomyces* genomes.

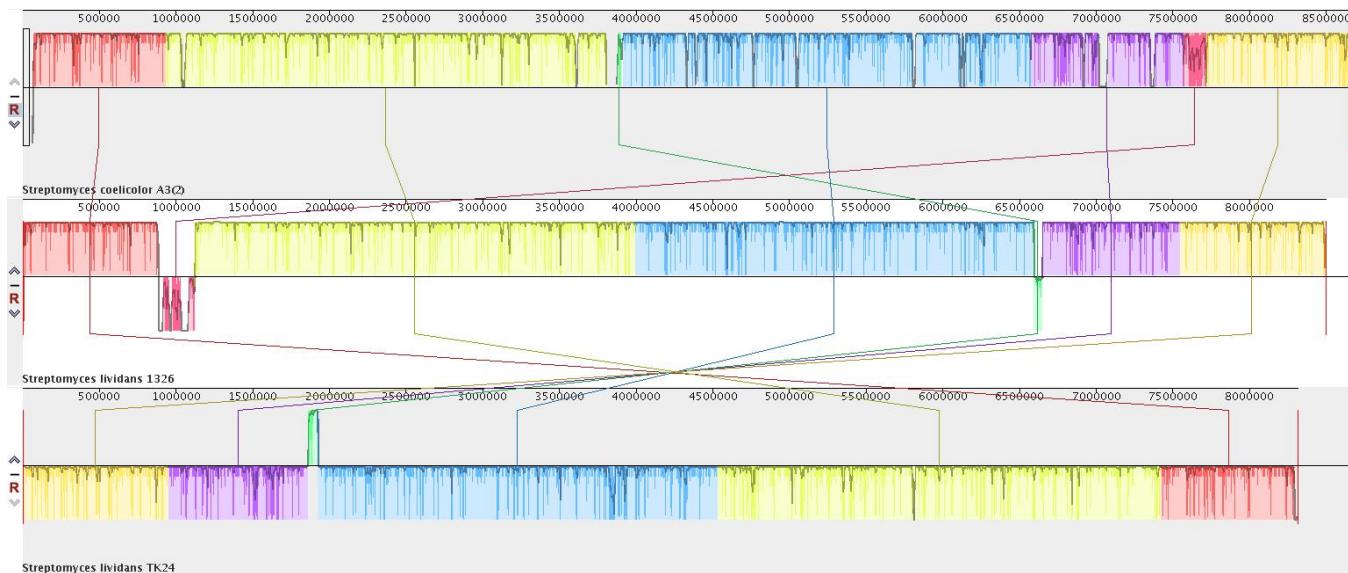


Figure S2: Visualization of the `progressiveMauve` alignment of the 3 *Streptomyces* genomes chosen for this analysis (from top to bottom): *S. coelicolor* AL645882, *S. lividans* NZ_CM001889, and *S. lividans* NZ_GG657756. Each coloured block represents a different locally co-linear block (LCB). Coloured lines connect LCBs that are similar between taxa. The black lines underneath each LCB represent the whole genome sequence of each of the *Streptomyces* taxa. Each LCB can be treated as a rearrangement, there have therefore been 6 rearrangements between these *Streptomyces* genomes.



Figure S3: This graph shows the time to complete a `progressiveMauve` alignment with varying numbers of *E. coli* genomes. The total number of genomes or taxa is along the x-axis and the total time in hours is along the right axis. Each black point represents data from one `progressiveMauve` alignment. All data points are connected by calculating locally estimated scatterplot smoothing (black line) with confidence intervals (grey band).

progressiveMauve Alignment

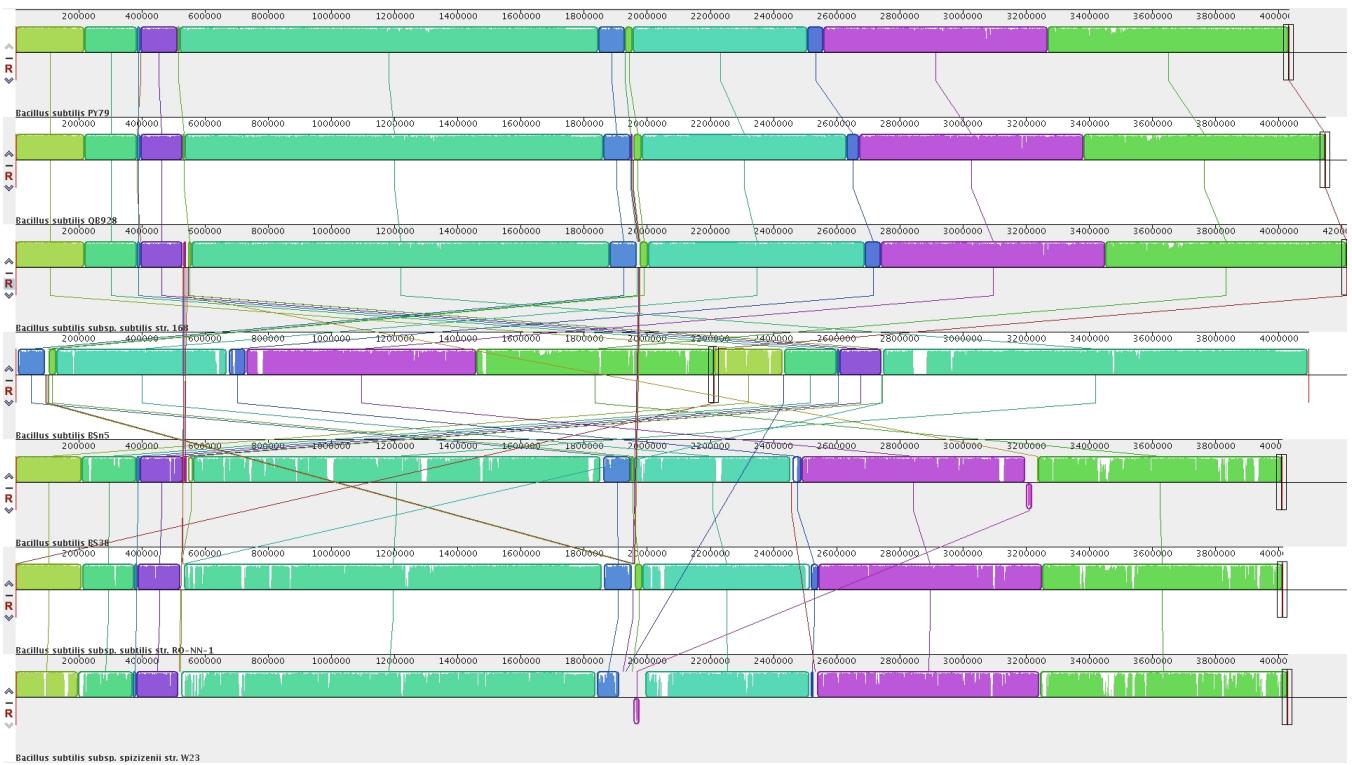


Figure S4: Visualization of the **progressiveMauve** alignment of the *B. subtilis* genomes. Each coloured block represents a different locally colinear block (LCB). Coloured lines connect LCBs that are similar between taxa. The black lines underneath each LCB represent the whole genome sequence of each of the *B. subtilis* taxa. From top to bottom the taxa are: *B. subtilis* PY79, *B. subtilis* QB928, *B. subtilis* 168, *B. subtilis* BSn5, *B. subtilis* BS38, *B. subtilis* RONN1, *B. subtilis* W23. Each LCB can be treated as a rearrangement, there have therefore been 12 rearrangements between these *B. subtilis* genomes.

Poor Sequence Alignment

After a re-alignment of **progressiveMauve** LCBs with **MAFFT** there were still regions of the alignment that were visibly poor. This prompted the additional alignment quality trimming using a custom Python script and **trimAl** (Capella-Gutiérrez et al. 2009). An example of what a “poor” alignment would look like can be found in Figure S5. The FASTA format of this segment of the alignment can be found on GitHub labelled as file “[poor_ecoli_alignment_example.fna](#)”.

This segment of **MAFFT** alignment (Figure S5) appears to have completely misaligned the second sequence (*E. coli* O157H7). When we look at the genes that these regions of DNA are found within (Table S2), we see that the second sequence (*E. coli* O157H7) does not have the same protein sequence as the other bacteria genes. Poor sequence alignments like this, as well as other non-homologous alignment regions were removed from the analysis. Please see the main paper for more detailed methods.

Alignment: poor_ecoli_alignment_example_TWO.fna
 Seaview [blocks=10 fontsize=10 A4] on Tue Mar 24 14:14:23 2020

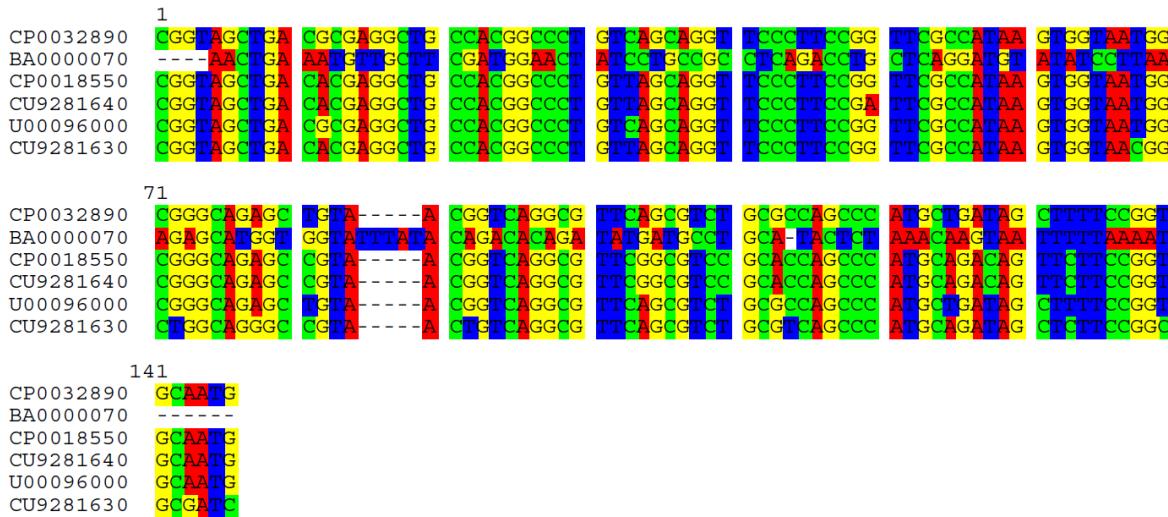


Figure S5: Visualization of a section of MAFFT alignment between the six strains of *E. coli*. This alignment was visualized with the SeaView graphical interface (Gouy et al. 2010).

<i>E. coli</i> Strain	NCBI Accession Number	Alignment Gene Id
0104H4	CP003289	O3K_04155
O157H7	BA000007	ECs3861
083H1	CP001855	NRG857_18350
IAI39	CU928164	yghE
K12	U00096	yghE
UMN026	CU928163	yghE

Table S2: *E. coli* strain, NCBI accession number, and Gene Id for the genes in the poor alignment example (Figure S5).

Phylogenetic Trees

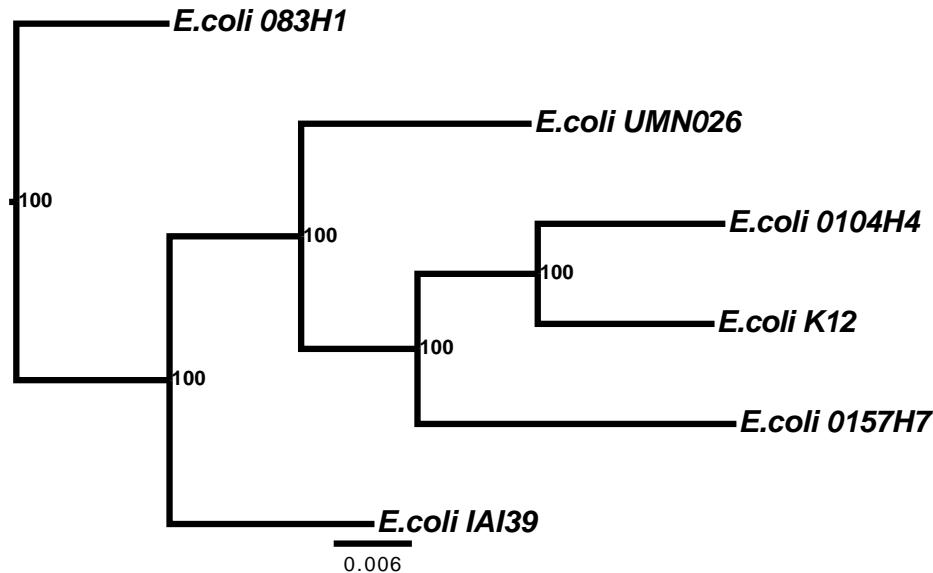


Figure S6: Phylogenetic tree of *E. coli* genomes. *Salmonella enterica* was used as an outgroup to root the tree. Branch lengths are to scale. The numbers at each node indicate the bootstrap value as a percentage. The number of bootstrapped trees was 100.

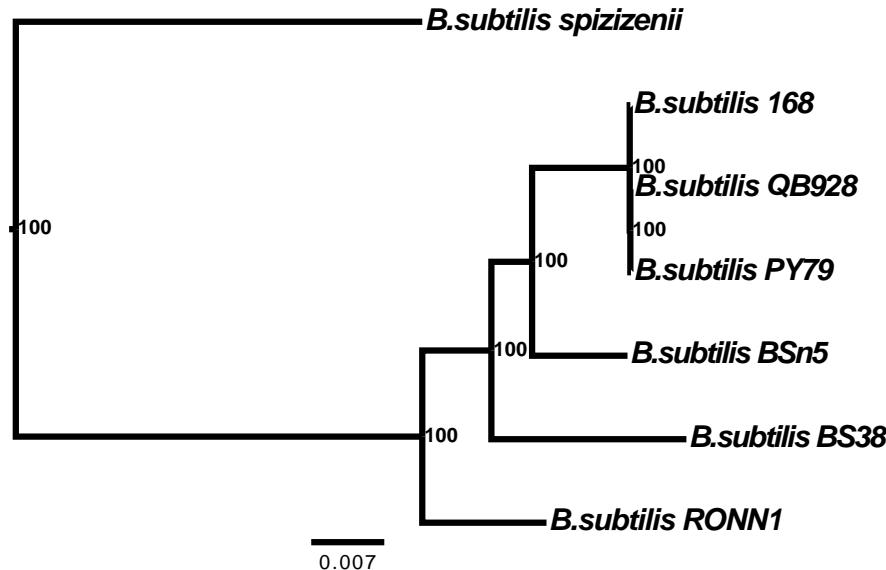


Figure S7: Phylogenetic tree of *B. subtilis* genomes. *Listeria monocytogenes* was used as an outgroup to root the tree. Branch lengths are to scale. The numbers at each node indicate the bootstrap value as a percentage. The number of bootstrapped trees was 100.

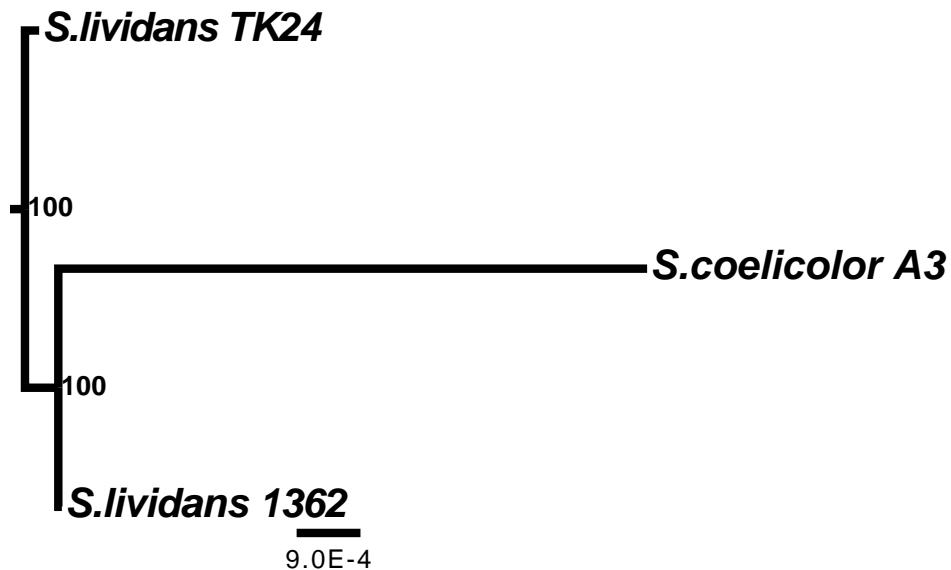


Figure S8: Phylogenetic tree of *Streptomyces* genomes. *Mycobacterium tuberculosis* was used as an outgroup to root the tree. Branch lengths are to scale. The numbers at each node indicate the bootstrap value as a percentage. The number of bootstrapped trees was 100.

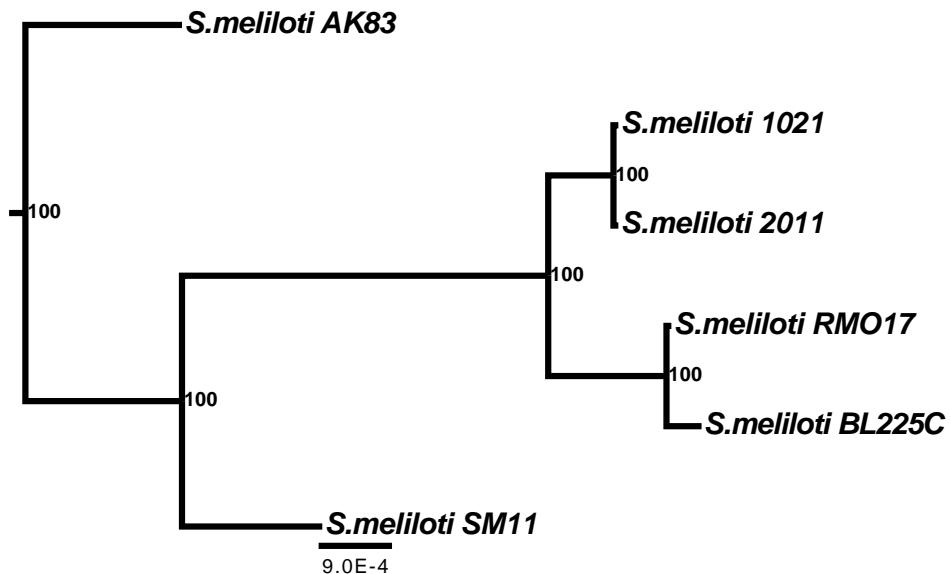


Figure S9: Phylogenetic tree using only the chromosomes of *S. meliloti*. *A. tumefaciens* circular chromosome was used as an outgroup to root the tree. Branch lengths are to scale. The numbers at each node indicate the bootstrap value as a percentage. The number of bootstrapped trees was 100.

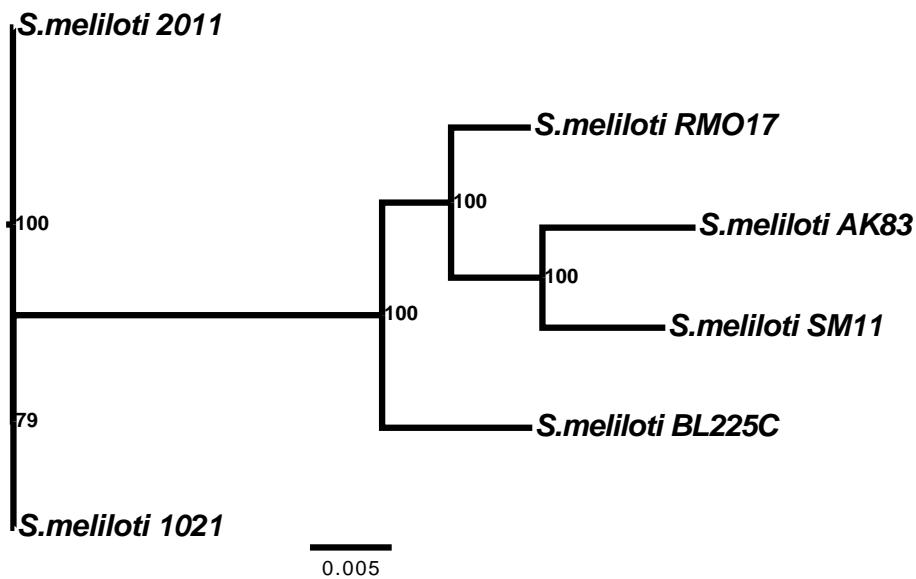


Figure S10: Phylogenetic tree using only pSymA of *S. meliloti*. *A. tumefaciens* circular plasmid was used as an outgroup to root the tree. Branch lengths are to scale. The numbers at each node indicate the bootstrap value as a percentage. The number of bootstrapped trees was 100.

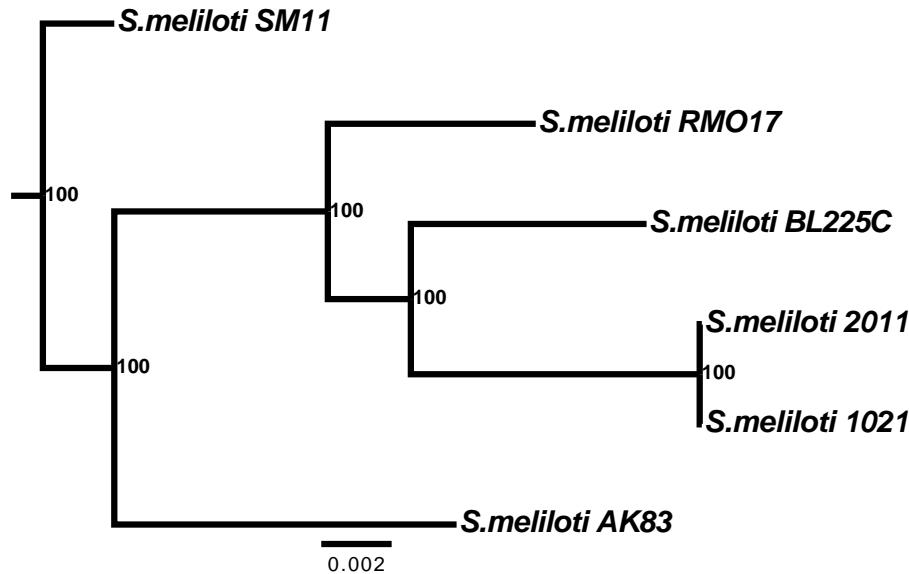


Figure S11: Phylogenetic tree using only pSymB of *S. meliloti*. *A. tumefaciens* circular chromid was used as an outgroup to root the tree. Branch lengths are to scale. The numbers at each node indicate the bootstrap value as a percentage. The number of bootstrapped trees was 100.

Bacteria Replicon	# of Total LCBs with Identical Tree	# of Total LCBs with Non- identical Tree	% of Total Alignment Discarded
<i>E. coli</i> Chromosome	30	7	25.44%
<i>B. subtilis</i> Chromosome	10	2	21.62%
<i>Streptomyces</i> Chromosome	NA	NA	NA
<i>S. meliloti</i> Chromosome	9	2	25.06%
<i>S. meliloti</i> pSymA	35	0	0%
<i>S. meliloti</i> pSymB	8	0	0%

Table S3: Number of Locally Colinear Blocks that had identical topologies to the “super sequence” tree, not identical to the “super sequence” tree, and the proportion of the total alignment that was represented by the non-identical tree topologies. Topologies that were not identical were determined to be different at the 5% significant value using an SH-test in RAxML (Stamatakis 2014). The SH-Test could not be preformed on *Streptomyces* as there were only three taxa present and RAxML needs a minimum of 4 taxa for the test (Stamatakis 2014).

Origin and Terminus Locations

Each of the bacterial strains used in this analysis vary in total genomic length, in some cases this difference is up to 856Kbp like in *E. coli* (Table S4). This will cause the farthest point from the origin of replication to appear larger because of the increased genome size of some strains.

Bacteria	Origin of Replication	Terminus of Replication	Length of Longest Genome (bp)
<i>E. coli</i>	3925744	1588773	5498450
<i>B. subtilis</i>	1	1942542	4215606
<i>Streptomyces</i>	3419363	1 & 8667507	8667507
<i>S. meliloti</i> Chromosome	1	1735626	3908022
<i>S. meliloti</i> pSymA	1350001	672888	1633319
<i>S. meliloti</i> pSymB	55090	896756	1690594

Table S4: Origin of replication and terminus of replication positions in replicons of *E. coli*, *B. subtilis*, *Streptomyces*, and *S. meliloti*. The origin and terminus of replication are values from the representative strain of each bacteria, which can be found in Supplementary Table S1. The linear nature of *Streptomyces* chromosome gives it two termini, one at each end of the chromosome. The lenght of the longest genome is the longest genome length from all strains/species of each bactiera. This is not necessarally the same as the genome length of the representative strain.

Origin Location	<i>E. coli</i> Chromosome	<i>B. subtilis</i> Chromosome	<i>Streptomyces</i> Chromosome	<i>S. meliloti</i> Chromosome	<i>S. meliloti</i> pSymA	<i>S. meliloti</i> pSymB
Moved 100kb Left	-1.445×10 ^{-7***}	4.374×10 ^{-9*}	6.909×10 ^{-9***}	-1.316×10 ^{-6***}	-1.058×10 ^{-6***}	-2.009×10 ^{-7***}
Moved 90kb Left	-1.544×10 ^{-7***}	-1.036×10 ^{-7***}	5.677×10 ^{-9***}	-1.32×10 ^{-6***}	-1.246×10 ^{-6***}	-1.357×10 ^{-7***}
Moved 80kb Left	-1.65×10 ^{-7***}	-1.072×10 ^{-7***}	8.11×10 ^{-9***}	-1.338×10 ^{-6***}	-1.398×10 ^{-6***}	-6.57×10 ^{-8***}
Moved 70kb Left	-1.667×10 ^{-7***}	-1.102×10 ^{-7***}	6.716×10 ^{-9***}	-1.363×10 ^{-6***}	-1.405×10 ^{-6***}	9.83×10 ⁻⁸
Moved 60kb Left	-1.64×10 ^{-7***}	-1.119×10 ^{-7***}	8.7×10 ^{-9***}	-1.324×10 ^{-6***}	-1.394×10 ^{-6***}	1.129×10 ^{-7***}
Moved 50kb Left	-1.446×10 ^{-7***}	-1.211×10 ^{-7***}	1.045×10 ^{-8***}	-1.36×10 ^{-6***}	-1.403×10 ^{-6***}	1.521×10 ^{-7***}
Moved 40kb Left	-1.4×10 ^{-7***}	-1.299×10 ^{-7***}	1.214×10 ^{-8***}	-1.255×10 ^{-6***}	-1.422×10 ^{-6***}	1.543×10 ^{-7***}
Moved 30kb Left	-1.498×10 ^{-7***}	-1.292×10 ^{-7***}	1.24×10 ^{-8***}	-1.26×10 ^{-6***}	-1.392×10 ^{-6***}	1.63×10 ^{-7***}
Moved 20kb Left	-1.51×10 ^{-7***}	-1.1×10 ^{-7***}	1.395×10 ^{-8***}	-1.525×10 ^{-6***}	-1.412×10 ^{-6***}	1.603×10 ^{-7***}
Moved 10kb Left	-1.262×10 ^{-7***}	-2.602×10 ⁻⁹	1.563×10 ^{-8***}	-1.599×10 ^{-6***}	-9.499×10 ^{-7***}	2.973×10 ^{-7***}
Moved 20kb Right	-1.305×10 ^{-7***}	-2.045×10 ^{-8***}	1.578×10 ^{-8***}	1.614×10 ^{-6***}	-1.026×10 ^{-6***}	3.505×10 ^{-7***}
Moved 20kb Right	-1.454×10 ^{-7***}	-1.006×10 ^{-7***}	1.903×10 ^{-8***}	-1.634×10 ^{-6***}	-1.475×10 ^{-6***}	1.649×10 ^{-7***}
Moved 30kb Right	-1.548×10 ^{-7***}	-8.596×10 ^{-8***}	2.046×10 ^{-8***}	-1.698×10 ^{-6***}	-1.417×10 ^{-6***}	1.526×10 ^{-7***}
Moved 40kb Right	-1.632×10 ^{-7***}	-8.378×10 ^{-8***}	2.125×10 ^{-8***}	-1.719×10 ^{-6***}	-1.367×10 ^{-6***}	1.589×10 ^{-7***}
Moved 50kb Right	-1.856×10 ^{-7***}	-7.879×10 ^{-8***}	1.957×10 ^{-8***}	-1.735×10 ^{-6***}	-1.277×10 ^{-6***}	1.654×10 ^{-7***}
Moved 60kb Right	-1.91×10 ^{-7***}	-6.98×10 ^{-8***}	1.974×10 ^{-8***}	-1.788×10 ^{-6***}	-1.169×10 ^{-6***}	1.645×10 ^{-7***}
Moved 70kb Right	-1.892×10 ^{-7***}	-6.634×10 ^{-8***}	1.934×10 ^{-8***}	-1.854×10 ^{-6***}	-1.059×10 ^{-6***}	1.843×10 ^{-7***}
Moved 80kb Right	-1.879×10 ^{-7***}	-5.814×10 ^{-8***}	2.313×10 ^{-8***}	-1.891×10 ^{-6***}	-9.07×10 ^{-7***}	1.90×10 ^{-7***}
Moved 90kb Right	-1.862×10 ^{-7***}	-4.314×10 ^{-8***}	2.304×10 ^{-8***}	-1.865×10 ^{-6***}	-7.171×10 ^{-7***}	2.415×10 ^{-7***}
Moved 100kb Right	-1.799×10 ^{-7***}	-2.597×10 ^{-8***}	1.945×10 ^{-8***}	-1.525×10 ^{-6***}	-6.572×10 ^{-7***}	3.095×10 ^{-7***}

Table S5: Logistic regression analysis of the number of substitutions along the genome of the respective bacterial replicons after the origin location was moved by the specified increments from the original origin of replication position (listed in Table S4). All results are marked with significance codes as followed: < 0.001 = ‘***’, 0.001 < 0.01 = ‘**’, 0.01 < 0.05 = ‘*’, 0.05 < 0.1 = ‘.’, > 0.1 = ‘ ’. Logistic regression was calculated after the origin of replication was moved to the new location in the genome and all subsequent positions were scaled around the origin accounting for bidirectionality of replication.

Bacteria Strain	Accession Number	Date Accessed
<i>E. coli</i> K12 Chromosome	U00096	September 26, 2016
<i>B. subtilis</i> 168 Chromosome	NC_000964	November 10, 2016
<i>S. coelicolor</i> A3 Chromosome	AL645882	November 30, 2016
<i>S. meliloti</i> Chromosome 1021	NC_003047	June 3, 2014
<i>S. meliloti</i> pSymA 1021	NC_003037	June 3, 2014
<i>S. meliloti</i> pSymB 1021	NC_003078	June 3, 2014

Table S6: Strains and species used for determining the protein coding regions of each bacterial replicon. GenBank reference annotation was used to determine all protein coding sections of the replicons. NCBI accession numbers and date accessed are provided.

Genomic Position Clustering

A custom R script was used to cluster genomic positions together based on a user specified genetic distance using single-link clustering. An illustration of the clustering method used in this supplemental test can be found in Figure S12. This clustering was done for genomic distances beginning at 1bp and increasing by one order of magnitude until 1,000,000bp difference exists between the taxa genomic positions. These newly clustered genomic positions were then put into the same substitution analysis as mentioned previously to determine the impact of this position clustering on the spatial substitution trends through a linear regression. A complete table of the statistical results from the clustering assessment are found in Table S7. The results from this analysis indicate that genomic positions up to 1,000,000bp apart can be considered a singular genomic position without altering the overall spatial substitution analysis.



Figure S12: Visualization of the genomic position clustering method. In this example, the user specified the genetic distance to be 2, all genomic positions within 2 base pairs would be clustered together. In this example we are looking at 6 taxa with genomic positions 10, 14, 12, 25, 22, and 20. Based on the clustering algorithm, positions 10, 14 and 12 would be grouped into a cluster (outlined in green), position 25 would be its own cluster (outlined in pink), and positions 22 and 20 would be grouped into another cluster (outlined in blue). Once the clusters are determined, a new genomic position for each of the clusters is calculated using the average of all positions within that cluster. In this example, the green cluster would have a new genomic position of 12 (the average between those three positions), the pink cluster would have the same genomic position of 25, and the blue cluster would have a new genomic position of 21. The new list of genomic positions for the 4 taxa would be: 12, 12, 12, 25, 21 and 21.

Position Difference	<i>E. coli</i> Chromosome	<i>B. subtilis</i> Chromosome	<i>Streptomyces</i> Chromosome	<i>S. meliloti</i> Chromosome	<i>S. meliloti</i> pSymA	<i>S. meliloti</i> pSymB
1bp	$-1.394 \times 10^{-7}***$	$-2.538 \times 10^{-8}**$	$1.736 \times 10^{-8}**$	$-1.541 \times 10^{-6}**$	$-9.130 \times 10^{-7}**$	$2.488 \times 10^{-7}***$
10bp	$-1.394 \times 10^{-7}***$	$-2.518 \times 10^{-8}***$	$-4.484 \times 10^{-9}***$	$-1.627 \times 10^{-6}***$	$-9.13 \times 10^{-7}***$	$3.487 \times 10^{-7}***$
100bp	$-1.764 \times 10^{-7}***$	$-1.417 \times 10^{-8}***$	$1.448 \times 10^{-8}***$	$-1.605 \times 10^{-6}***$	$-1.166 \times 10^{-6}***$	$4.021 \times 10^{-7}***$
1000bp	$-1.784 \times 10^{-7}***$	$-1.417 \times 10^{-8}***$	$1.505 \times 10^{-8}***$	$-1.605 \times 10^{-6}***$	$-1.153 \times 10^{-6}***$	$4.021 \times 10^{-7}***$
10000bp	$-1.712 \times 10^{-7}***$	$-3.496 \times 10^{-8}***$	$4.790 \times 10^{-8}***$	$-1.605 \times 10^{-6}***$	-3.570×10^{-8}	$3.784 \times 10^{-7}***$
100000bp	$-2.061 \times 10^{-7}***$	$-3.561 \times 10^{-8}***$	$4.167 \times 10^{-9}***$	$-1.605 \times 10^{-6}***$	$-4.676 \times 10^{-7}***$	$3.784 \times 10^{-7}***$
1000000bp	$4.229 \times 10^{-8}***$	$-7.710 \times 10^{-9}***$	$6.083 \times 10^{-8}***$	$-1.605 \times 10^{-6}***$	$4.285 \times 10^{-6}***$	$-8.888 \times 10^{-7}***$

Table S7: Results from the position clustering analysis. Logistic regression analysis of the number of substitutions along the genome of the respective bacteria replicons to test position differences. The “Position Difference” column denotes different base pair distances that the positions in the genome were clustered together as. All results are marked with significance codes as followed: $< 0.001 = ***$, $0.001 < 0.01 = **$, $0.01 < 0.05 = *$, $0.05 < 0.1 = .$, $> 0.1 = '$. Logistic regression was calculated after the positions in the genome were determined to be the same at each position difference listed in the first column.

Bacteria and Replicon	Average Replicon Length	Number of Sites	Number of Substitutions
<i>E. coli</i> Chromosome	5082529	2318259	353740
<i>B. subtilis</i> Chromosome	4077077	2032176	185060
<i>Streptomyces</i> Chromosome	8494093	6057063	24046
<i>S. meliloti</i> Chromosome	3426881	1892874	11210
<i>S. meliloti</i> pSymA	1455940	571278	13132
<i>S. meliloti</i> pSymB	1664597	1248879	28941

Table S8: Total number of protein coding sites in each replicon for this analysis and the number of those sites that have a substitution (multiple substitutions at one site are counted as two substitutions).

High Substitutions Gene Example

Throughout this analysis there are a few genes/gene segments in all the bacterial replicons that have relatively high numbers of substitutions when compared to other genes or gene segments. These high numbers of substitutions are indeed real changes seen in homologous genes. To illustrate this, we have chosen a segment of alignment from *Streptomyces*. Information about the genes involved in this segment can be found in Table S9. A protein alignment for these genes can be found on GitHub (https://github.com/dlato/Spatial_Patterns_of_Substitutions) under the file name “*Streptomyces_high_substitutions_gene_example.txt*”.

Both *S. lividans* strains have 100% sequence identity at the DNA level, while the *S. coelicolor* species has 87.2% sequence identity with the *S. lividans* strains for this particular alignment. Despite this high sequence identity and almost identical protein alignment (Figures S13 and S14), there are a total of 31 substitutions (across all nodes of the phylogenetic tree, Figure S8) within this short stretch of sequence. It is segments like these that are resulting in the appearance of extremely high numbers of substitutions in sections of all the bacterial repliconic genomes.

Species	NCBI Acession Number	Gene Id
<i>S. coelicolor</i> A3	AL645882	SCO6334
<i>S. lividans</i> 1362	NZ_CM001889	SLI_RS32020
<i>S. lividans</i> TK24	NZ_GG657756	SSPG_RS06405

Table S9: Information about the example gene segment with high number of substitutions.

Alignment: *Streptomyces_high_substitutions_gene_example.txt*
Seaview [blocks=10 fontsize=10 A4] on Thu Apr 30 13:24:40 2020

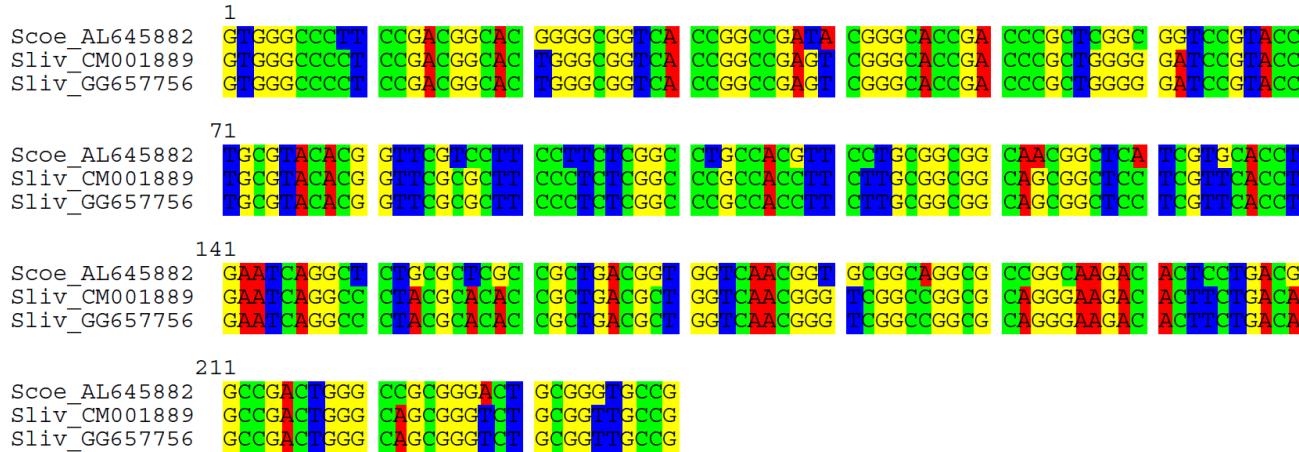


Figure S13: Visualization of the nucleotide alignment of *Streptomyces* genes with high numbers of substitutions. Alignment visualization was performed with SeaView (Gouy et al. 2010)

Alignment: PROTEIN_ALN.txt
 Seaview [blocks=10 fontsize=10 A4] on Mon Mar 9 15:02:33 2020

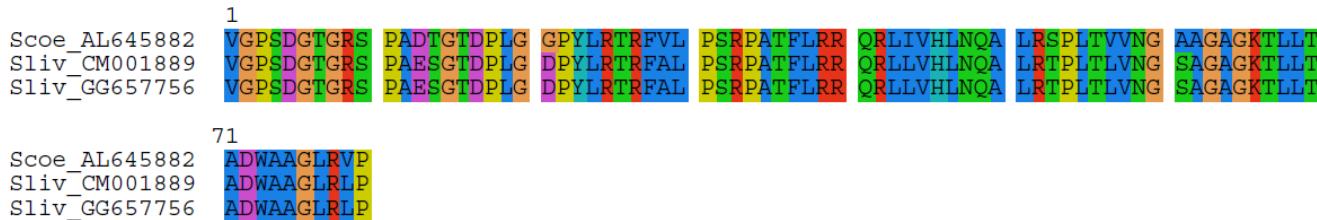


Figure S14: Visualization of the protein alignment of *Streptomyces* genes with high numbers of substitutions. Alignment visualization was performed with SeaView (Gouy et al. 2010)

High Substitution Distribution

Bacteria and Replicon	Bidirectional Genomic Position (bp)	Protein/Gene Examples
<i>E. coli</i> Chromosome	1130000 - 1140000	Uncharacterized protein
		Hypothetical protein
		Lipoprotein
		Transcriptional activator
	1720000 - 1740000	Hypothetical protein
		Predicted protein
		Small toxic polypeptide
<i>B. subtilis</i> Chromosome	560000 - 570000	Hypothetical protein
		Derived by automated computational analysis
		Membrane protein
	1820000 - 1380000	Derived by automated computational analysis
<i>Streptomyces</i> Chromosome	3550000 - 3570000	Hypothetical protein
		Derived by automated computational analysis
		Putative integral membrane protein
		Reductase
<i>S. meliloti</i> Chromosome	80000 - 90000	Hypothetical proteins
	730000 - 740000	Hypothetical proteins
		Putative proteins
<i>S. meliloti</i> pSymA	100000 - 110000	Hypothetical proteins
	800000 - 810000	Hypothetical protein
		Transporter protein
<i>S. meliloti</i> pSymB	450000 - 460000	Hypothetical protein
		Putative oxidoreductase
		Hypothetical proteins
	610000 - 620000	Hypothetical protein
		Putative transport regulator
		Predicted membrane protein

Table S10: Table of high number of substitutions per 10Kbp genomic regions for each bacterial replicon and examples of the associated proteins/gene functions found in that region. The genomic position begins at the origin of replication and continues in both directions until the terminus of replication (bidirectional replication).

Weighted, Non-weighted, and 20Kbp Near and Far From the Origin Substitution Linear Regression Analysis

Bacteria and Replicon	Protein Coding	
	Weighted	Non-Weighted
<i>E. coli</i> Chromosome	$-2.35 \times 10^{-10}***$	$-2.84 \times 10^{-4}***$
<i>B. subtilis</i> Chromosome	$-7.96 \times 10^{-10}**$	$-1.73 \times 10^{-4}**$
<i>Streptomyces</i> Chromosome	$2.38 \times 10^{-11}*$	NS
<i>S. meliloti</i> Chromosome	$-9.26 \times 10^{-11}***$	$-2.21 \times 10^{-5}***$
<i>S. meliloti</i> pSymA	NS	NS
<i>S. meliloti</i> pSymB	NS	NS

Table S11: Linear regression on 10kb sections of the genome with increasing distance from the origin of replication after accounting for bidirectional replication. Weighted columns have the total number of substitutions in each 10kb section of the genome divided by the total number of protein coding sites in the genome. Non-weighted columns are performing a linear regression on the total number of substitutions in each 10kb section of the genome. All results are marked with significance codes as followed: $< 0.001 = ***$, $0.001 < 0.01 = **$, $0.01 < 0.05 = *$, $> 0.05 = \text{NS}$.

Bacteria and Replicon	Protein Coding			
	Correlation Coefficient		Number of Substitutions per 20kb Near	
	20kb Near		Origin	Terminus
<i>E. coli</i> Chromosome	$1.47 \times 10^{-5}**$	$4.91 \times 10^{-6}**$	5.97×10^{-3}	5.73×10^{-3}
<i>B. subtilis</i> Chromosome	$-4.64 \times 10^{-6}**$	$1.46 \times 10^{-5}***$	3.01×10^{-3}	5.40×10^{-3}
<i>Streptomyces</i> Chromosome	NS	$7.48 \times 10^{-4}***$	6.43×10^{-4}	1.95×10^{-3}
<i>S. meliloti</i> Chromosome	$8.11 \times 10^{-5}**$	NS	9.76×10^{-5}	6.10×10^{-5}
<i>S. meliloti</i> pSymA	NS	$-3.40 \times 10^{-5}***$	9.58×10^{-4}	3.55×10^{-3}
<i>S. meliloti</i> pSymB	NS	$-5.72 \times 10^{-5}***$	2.05×10^{-3}	1.27×10^{-3}

Table S12: Logistic regression on 20kb closest and farthest from the origin of replication after accounting for bidirectional replication and outliers. Number of substitutions was calculated by taking the total number of substitutions in each of the 20Kbp regions and dividing by the total number of sites in those regions. All results are marked with significance codes as followed: $< 0.001 = ***$, $0.001 < 0.01 = **$, $0.01 < 0.05 = *$, $> 0.05 = \text{NS}$.

Multiple linear regressions were performed to determine if there was any correlation between number of substitutions and distance from the origin of replication. A linear regression to determine how the weighted total number of substitutions per 10Kbp section of the genome changes with genomic position was performed (Table S11). All additional linear regression results (Table S11) mirror the results from the logistic regression on presence or absence of substitutions and changes in genomic position (see the Main Paper results section for more information). The results from these supplemental tests are consistent with the results from the linear regression found in the Main Paper, most bacterial replicons have a decreasing number of substitutions when moving away from the origin of replication.

To calculate the non-weighted values of the total number of substitutions per 10Kbp region of the genome, the total number of substitutions was summed up over each 10Kbp region of the genome while accounting for bidirectional replication (see Main Paper for details). A linear regression on these total number of substitutions in each 10kb section of the genome was performed to see how the number of substitutions changes with distance from the origin of replication. The weighted values of the total number of substitutions per 10Kbp region of the genome, the total number of substitutions was summed up over each 10Kbp region of the genome while accounting for bidirectional replication (see Main Paper for details). These summed values were then divided by the total number of protein coding sites in each 10Kbp region to obtain the weighted value. A linear regression on these weighted total number of substitutions in each 10kb section of the genome was performed to see how the number of substitutions changes with distance from the origin of replication.

We took a closer look at 20Kbp regions of the replicons close and far from the origin of replication. We performed a logistic regression on the presence or absence of a substitution with distance from the origin of replication. Data points from the 20Kbp regions closest to the origin of replication and data points from the 20Kbp regions closest to the terminus of replication were used for this portion of the analysis. Outliers were removed from this analysis. The number of substitutions per site was also calculated in each of these 20Kbp regions for each bacterial replicon. We were unable to determine a consistent spatial substitution trend when considering only the 20Kbp near and far from the origin of replication in all bacterial replicons. Some bacterial replicons had a positive correlation coefficient, indicating that the number of substitutions increases with increasing distance from the origin of replication (Table S12). Other replicons had a negative correlation coefficient, suggesting that the number of substitutions decreases with increasing distance from the origin of replication (Table S12). Additionally, it was unclear if the number of substitutions per site locally were higher near the origin of replication or near the terminus. Some bacteria had higher number of substitutions per site near the origin (*E. coli*, *S. meliloti* chromosome and pSymB), while other replicons has the opposite trend (*B. subtilis*, *Streptomyces* and *S. meliloti* pSymA) (Table S12). These results suggest that on a small local scale, there are varying patterns of substitutions with respect to distance from the origin of replication. This varies between bacteria, and in some cases even within the same bacteria (*E. coli*). This variation locally does not allow us to make any overarching statements about the local distribution of substitutions in bacterial genomes. It is therefore more useful to consider the global (genome wide) pattern of substitutions when making overarching statements about genomic substitution arrangements.

Total Number of Sites Linear Regression

We performed a linear regression on the total number of protein coding sites and distance from the origin of replication. We found that the total number protein coding sites decreases with distance from the origin of replication in majority of the bacterial replicons in this analysis. We were unable to detect a significant relationship between the number of protein coding sites and distance from the origin of replication in pSymB of *S. meliloti*.

Bacteria and Replicon	Coefficient Estimate
<i>E. coli</i> Chromosome	$-4.09 \times 10^{-2} ***$
<i>B. subtilis</i> Chromosome	$-1.93 \times 10^{-2} **$
<i>Streptomyces</i> Chromosome	$-1.24 \times 10^{-3} ***$
<i>S. meliloti</i> Chromosome	$-7.85 \times 10^{-2} ***$
<i>S. meliloti</i> pSymA	$-2.49 \times 10^{-2} *$
<i>S. meliloti</i> pSymB	NS

Table S13: Linear regression analysis of the total number of protein coding sites per 10kb along the genome of the respective bacteria replicons. Linear regression was calculated after the origin of replication was moved to the beginning of the genome and all subsequent positions were scaled around the origin accounting for bidirectionality of replication. All results are marked with significance codes as followed: $< 0.001 = '***'$, $0.001 < 0.01 = '**'$, $0.01 < 0.05 = '*'$, $> 0.05 = 'NS'$.

Average dN , dS , and ω per Gene Values

The average dN , dS , and ω values per gene were calculated. For genes that were split into multiple parts (due to the presence of gaps or poor homology in the alignment), the dN , dS , and ω values for each gene part were averaged to obtain a single average value per gene. A complete list of these values can be found on GitHub (www.github.com/dlato/Spatial_Patterns_of_Substitutions) under the file name “Supplementary_table_per_gene_dN_dS_omega.pdf”.

Distribution of dN , dS , and ω

Bacteria and Replicon	Outliers (%)	Zero Value (%)		
		dN	dS	ω
<i>E. coli</i> Chromosome	7.13	14.33	1.19	14.33
<i>B. subtilis</i> Chromosome	5.33	4.09	0.09	4.09
<i>Streptomyces</i> Chromosome	8.71	49.66	28.52	49.65
<i>S. meliloti</i> Chromosome	16.20	65.58	66.72	65.58
<i>S. meliloti</i> pSymA	6.90	11.39	9.45	11.39
<i>S. meliloti</i> pSymB	6.06	13.01	5.16	13.01

Table S14: Percent of data that was calculated to be an outlier or had a selection variable (dN , dS , and ω) value of zero.

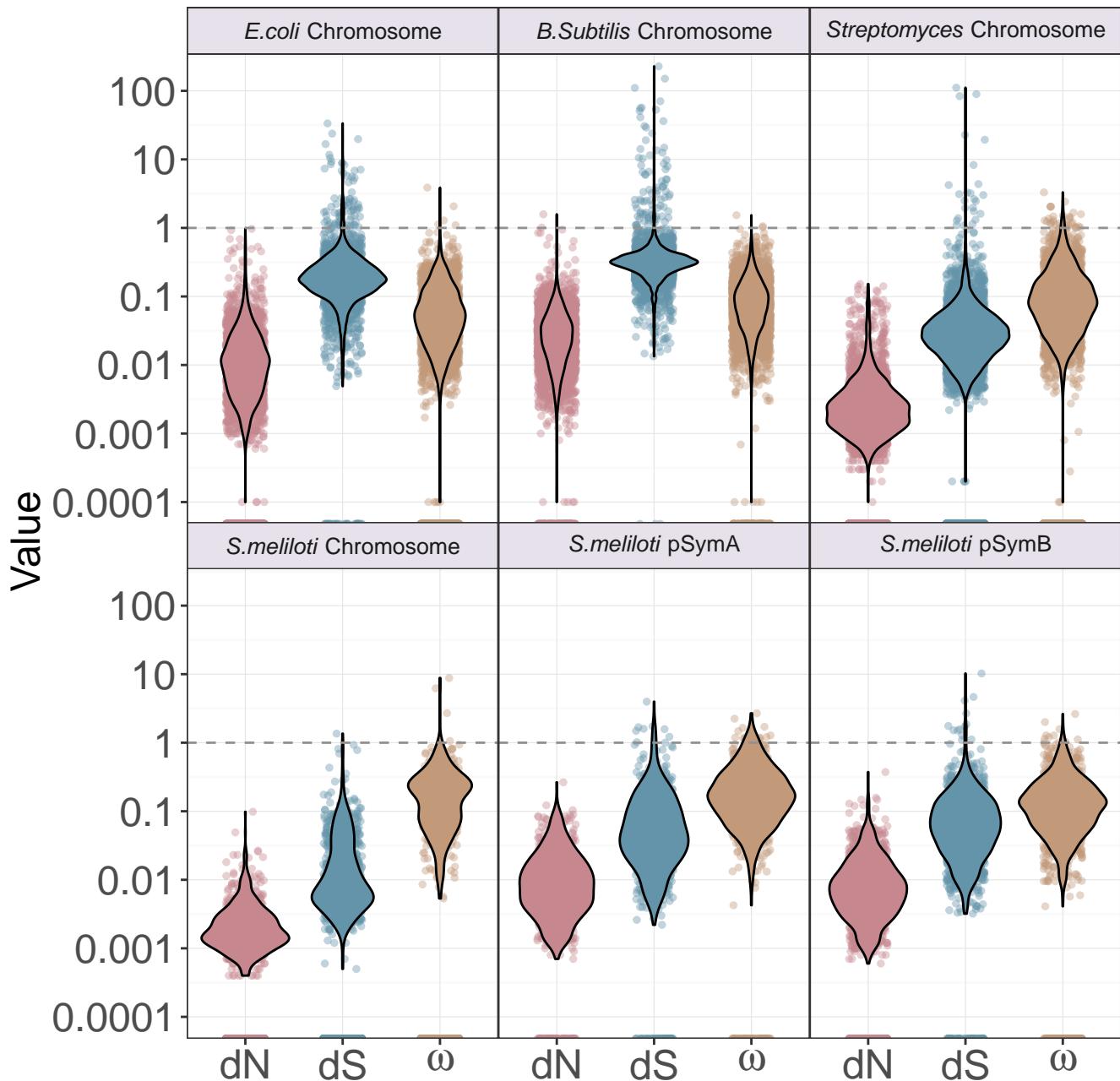


Figure S15: Distribution of all dN , dS , and ω values on a log base 10 scale for each replicon. Individual points are shown as a strip chart (which has been jittered in the x-direction in R (Wickham et al. 2019)), and the density of these selection values is shown in the overlaid violin plot. All points are included in this graphic including outliers. For more information on how outliers were calculated, please see the main paper. Any dN , dS , or ω values that had a value of zero is pushed to the bottom of the x-axis. Since these values will not appear on a log base 10 scale, they are not included in the violin portions of this graphic. For a complete list of zero values in each of the selection categories please refer to Table S14. In these graphs there is a horizontal line of values at 0.0001 for most of the selection coefficients in most of the bacterial replicons. This is due to rounding practices when `codeml` (Yang 1997) calculates dN , dS , and ω values.

20Kbp Near and Far From Origin Selection Linear Regression Analysis

We additionally took a closer look at 20 genes close and far from the origin of replication. We performed a linear regression on the change in selection values (dN , dS , and ω) with distance from the origin of replication in these genes (Table S15). For majority of the bacterial replicons we failed to find a trend, which is not surprising since there was no evidence of an overall genomic trend when looking at these values (see Main Paper for results). Again, we are unable to conclude that there is a consistent overall trend for any of the selection values, dN , dS , and ω .

Bacteria and Replicon	Near Origin			Near Terminus		
	dN	dS	ω	dN	dS	ω
<i>E. coli</i> Chromosome	NS	NS	NS	$3.13 \times 10^{-7}*$	NS	NS
<i>B. subtilis</i> Chromosome	NS	NS	NS	NS	NS	NS
<i>Streptomyces</i> Chromosome	$-1.35 \times 10^{-7}**$	NS	NS	NS	NS	NS
<i>S. meliloti</i> Chromosome	NS	NS	NS	NS	NS	NS
<i>S. meliloti</i> pSymA	NS	NS	NS	$-2.53 \times 10^{-7}*$	NS	NS
<i>S. meliloti</i> pSymB	NS	NS	NS	NS	$4.92 \times 10^{-6}*$	NS

Table S15: Linear regression for dN , dS , and ω calculated for each bacterial replicon for the 20 genes closest and 20 genes farthest from the origin of replication. All results are marked with significance codes as followed: p: $< 0.001 = '***'$, $0.001 < 0.01 = '**'$, $0.01 < 0.05 = '*'$, $> 0.05 = 'NS'$.

S. meliloti Chromosome Selection Analysis Without Outliers

Due to the extremely high sequence similarity of the *S. meliloti* chromosomes in this analysis, there are a relatively low number of substitutions and therefore many dN and ω values that are equal to zero (see Table S14). The high number of zero values were included in the original calculation of outliers (see Main Paper for more details) causing all of the non-zero dN and ω values to be classified as outliers (see Figure 6 in the Main Paper). We decided to perform the same calculations on dN , dS , and ω but including the outliers to see what the results would have been. A visualization of the distribution of dN , dS , and ω along the chromosome of *S. meliloti* is seen in Figure S16. The average values for dN , dS , and ω are found in Table S16 and the linear regression to determine if there is a correlation between distance from the origin of replication and dN , dS , and ω values for the chromosome of *S. meliloti* is found in Table S17. We also looked at the values of dN , dS , and ω in the 20Kbp regions near and far from the origin of replication (including outliers) in the *S. meliloti* chromosome, these results are summarized in Table S18. The methods for these calculations are the same as in the Main Paper and in section 20Kbp Near and Far From Origin Selection Linear Regression Analysis, however, outliers were not removed from these calculations.

The results in Tables S16 - S18 closely reflect the results of the *S. meliloti* analysis when outliers were included (see Main Paper). We were unable to determine a significant correlation between dN and ω values and distance from the origin of replication. The significant correlation between dS and distance from the origin of replication is small. Even when the outliers (non-zero values of dN and ω) are included in the selection analysis, we still can find no evidence for an overall trend between distance from the origin of replication and dN , dS , and ω values.

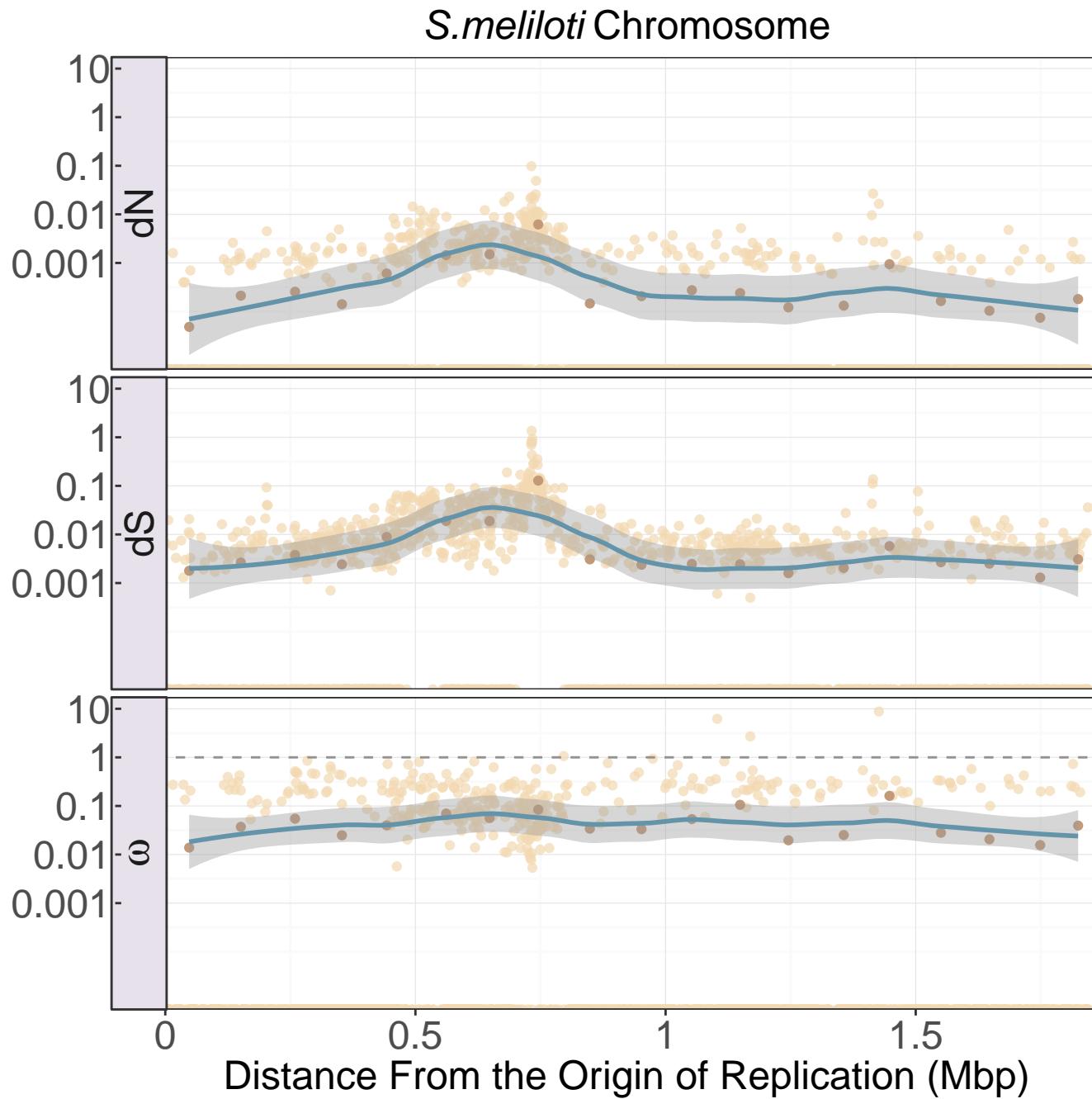


Figure S16: The graph show the values of dN , dS , and ω along the chromosome of *S. meliloti*. Distance from the origin of replication is on the x-axis beginning with the origin of replication denoted by position zero on the left, and the terminus indicated on the far right. The y-axis of the graph indicates the value of dN , dS , and ω found at each gene segment position of the chromosome. Outliers are included in this graph. The average dN , dS , and ω values for each 10,000bp regions of the replicon were calculated and represented by the dark brown points. A trend line represented in blue (using the `loess` method), was fit to these average values and the associated 95% confidence intervals for this line is represented by the grey ribbon around the blue trend line. For a complete list zero value information, please see Table S14.

Genome Average			
Bacteria and Replicon	dS	dN	ω
<i>S. meliloti</i> Chromosome	0.0100	0.0007	0.0677

Table S16: Weighted averages of dN , dS , and ω values calculated for *S. meliloti* chromosome using the gene length as the weight. Arithmetic mean was calculated for the per gene averages for *S. meliloti* chromosome. Outliers were included in the calculation.

Bacteria and Replicon	dN	dS	ω
<i>S. meliloti</i> Chromosome	NS (-1.96×10^{-10})	$-2.29 \times 10^{-9}*$	NS (5.96×10^{-9})

Table S17: Linear regression to determine the correlations between dN , dS , and ω values and distance from the origin of replication. Outliers were included in the calculation. All results are marked with significance codes as followed: p: $< 0.001 = '***'$, $0.001 < 0.01 = '**'$, $0.01 < 0.05 = '*'$, $> 0.05 = 'NS'$.

Bacteria and Replicon	Near Origin			Near Terminus		
	dN	dS	ω	dN	dS	ω
<i>S. meliloti</i> Chromosome	NS (2.16×10^{-8})	NS (-7.05×10^{-8})	NS (3.07×10^{-6})	NS (-1.50×10^{-8})	NS (2.22×10^{-7})	NS (-2.06×10^{-6})

Table S18: Linear regression for dN , dS , and ω calculated for the 20 genes closest and 20 genes farthest from the origin of replication in the *S. meliloti* chromosome. Outliers were included in the calculation. All results are marked with significance codes as followed: p: $< 0.001 = '***'$, $0.001 < 0.01 = '**'$, $0.01 < 0.05 = '*'$, $> 0.05 = 'NS'$.

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