

Research

Asymmetric directional mutation pressures in bacteria

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Abstract

Background: When there are no strand-specific biases in mutation and selection rates (that is, in the substitution rates) between the two strands of DNA, the average nucleotide composition is theoretically expected to be $A = T$ and $G = C$ within each strand. Deviations from these equalities are therefore evidence for an asymmetry in selection and/or mutation between the two strands. By focusing on weakly selected regions that could be oriented with respect to replication in 43 out of 51 completely sequenced bacterial chromosomes, we have been able to detect asymmetric directional mutation pressures.

Results: Most of the 43 chromosomes were found to be relatively enriched in G over C and T over A, and slightly depleted in G+C, in their weakly selected positions (intergenic regions and third codon positions) in the leading strand compared with the lagging strand. Deviations from $A = T$ and $G = C$ were highly correlated between third codon positions and intergenic regions, with a lower degree of deviation in intergenic regions, and were not correlated with overall genomic G+C content.

Conclusions: During the course of bacterial chromosome evolution, the effects of asymmetric directional mutation pressures are commonly observed in weakly selected positions. The degree of deviation from equality is highly variable among species, and within species is higher in third codon positions than in intergenic regions. The orientation of these effects is almost universal and is compatible in most cases with the hypothesis of an excess of cytosine deamination in the single-stranded state during DNA replication. However, the variation in G+C content between species is influenced by factors other than asymmetric mutation pressure.

Background

The G+C content of bacterial DNA ranges from around 25% to around 75% [1,2]. In contrast to the nuclear genomes of some vertebrates, the intragenomic heterogeneity of G+C content is generally small in bacteria [3,4], although there are some exceptions [5-7]. The wide interspecies variation and narrow intraspecies heterogeneity of the DNA G+C content was interpreted to be the result of differences in the

bidirectional mutation rates between AT and GC pairs [8]. This kind of mutation pressure is said to be the G+C pressure. From Watson-Crick base-pairing rules [9], the G+C content is exactly the same for the two complementary strands in a DNA duplex, so the G+C pressure effects are symmetric with respect to the two strands. The effects of G+C pressure are dramatically increased in regions where selective pressure is weak: for instance, in the third codon

positions in protein-coding sequences, where most substitutions do not alter the encoded amino acid, the average G+C content ranges from at least 7% to 95% between bacteria of different species [7,10,11]. It should be stressed that symmetric directional mutation effects are not restricted to purely neutral regions, and strongly influence amino-acid frequencies in proteins [12-16].

The two strands of a DNA duplex must have the same G+C content, but the different bases can still vary in frequency. For instance, one strand may have more G than C, say $G/(G+C) = 0.8$, and by complementarity the other one will have more C than G, $G/(G+C) = 0.2$ in this case. The strand-specific asymmetries in DNA have been the subject of considerable research [17-23]. Briefly, the theoretical predictions are as follows: when mutation and selection are assumed to be symmetric with respect to the two strands of DNA, parity rule 1 (PR1) holds where the following pairs of substitution rates are equal: $r_{AT} = r_{TA}$, $r_{GC} = r_{CG}$, $r_{AG} = r_{TC}$, $r_{GA} = r_{CT}$, $r_{AC} = r_{TG}$, $r_{CA} = r_{GT}$ [20]. Here, r_{AT} , and so on, are substitution rates of $A \rightarrow T$, and so on, in a specific strand. Under the PR1 hypothesis, the intrastrand base composition at equilibrium is expected to be always in a particular state, the parity rule 2 (PR2) state, such that $A = T$ and $G = C$ within each strand [20,21,24]. This PR2 state is more than just an equilibrium point, because convergence towards PR2 continues even if the substitution rates are modified: there is no way to escape from the PR2 state under the PR1 hypothesis [25]. However, a bias in mutation or selection between the two strands of DNA can generate deviations from PR2 (that is, $A \neq T$, or $G \neq C$, or both). In this situation, the underlying cause, regardless of its mutational or selective nature, is asymmetric between the two strands.

Note that there is a serious confusion in the literature about substitution matrices that are 'symmetric' in the sense that the two strands evolve with the same pattern of substitutions (PR1 hypothesis), and 'symmetric' in the sense that the terms above and below the diagonal are the same (the SYM model, where $r_{ij} = r_{ji}$). The SYM model adopted previously by others, especially in the field of molecular phylogeny [26], assumes the equality of intrastrand mutation rates between any two bases: in contrast to the PR1 model, the SYM model does not incorporate DNA base-pairing rules. The SYM model has long been recognized as unrealistic because it predicts $A = T = G = C$ at equilibrium without biases. The PR1 model is not a special case of the SYM model; rejection of the SYM model does not lead to rejection of the PR1 model.

The effect of asymmetric mutation pressure on the amino-acid content of proteins has been reported [27-29]. In terms of amino-acid composition, the diversifying selection on integral membrane proteins (enriched in hydrophobic amino acids) and on cytoplasmic proteins (enriched in hydrophilic amino acids) is generally the most important factor in within-proteome variability [30]. In *Borrelia burgdorferi*,

however, the effects of this selective pressure are quantitatively less important than those resulting from asymmetric mutation pressure [31], stressing again that mutation pressure is far from being a negligible phenomenon.

Deviations from PR2 have been studied using many different approaches, including correspondence discriminant analysis [32], linear discriminant analysis with rotating windows [28], correspondence analysis applied to relative synonymous codon usage [27,33,34], analysis of variance [35], a comparative genomics approach [22,36-38], simple DNA walks [39], detrended DNA walks split by codon positions [40-42], skewed oligomer distributions [43], and moving windows plots with direct [44,45] or cumulated [46,47] or codon position-dependent [48] skew indices. Here we have used a simple graphical representation, very close to the raw data, that provides a straightforward visualization and interpretation of results.

Consider a complete annotated bacterial chromosome in which origin and terminus of replication have been located so that we know whether a subsequence is on the leading strand or the lagging strand at replication (Figure 1). Let A_3 , C_3 , G_3 , and T_3 denote the base counts in the sense strand of a given coding sequence when only the weakly selected third codon positions are considered. Deviations from PR2 in third codon positions are characterized by $G_3/(G_3+C_3)$ and $A_3/(A_3+T_3)$ to draw PR2-plots [7].

Figure 2a shows diagrammatically what would be expected under the null hypothesis PR1: the PR2 state should be observed. Although there will be statistical fluctuations, spreading genes across the space indicated by each circle, the circles are centered at the midpoint of the plot (0.5, 0.5) which corresponds to the PR2 state. The range of dispersion is greater for the leading-strand group, because we have assumed here (for clarity, to avoid overlap in the figures), that there is an excess of coding sequences in the leading strand, as is usually observed in bacteria [48]. Figure 2b shows diagrammatically what would be expected when replication-associated mutation pressure enriches the leading strand in G and T. The leading-strand coding sequences, whose sense strand is, by definition, the leading strand for replication (Figure 1) are therefore also enriched in G and T. Thus, x_1 , their average $G_3/(G_3+C_3)$ value, is greater than 0.5, and y_1 , their average $A_3/(A_3+T_3)$ value, is less than 0.5. From DNA duplex base-pairing rules, if the leading strand for replication is enriched in G and T, its complementary lagging strand is conversely enriched in C and A. Therefore, we have a perfectly symmetric situation for the lagging-strand coding sequences, whose sense strand is, by definition, the lagging strand for replication (Figure 1), with $x_2 = 1 - x_1$ and $y_2 = 1 - y_1$. Note that the overall average is biased towards the leading-strand group because of the excess of leading-strand coding sequences, but that the midpoint ($x_c = (x_1 + x_2)/2$, $y_c = (y_1 + y_2)/2$) is not

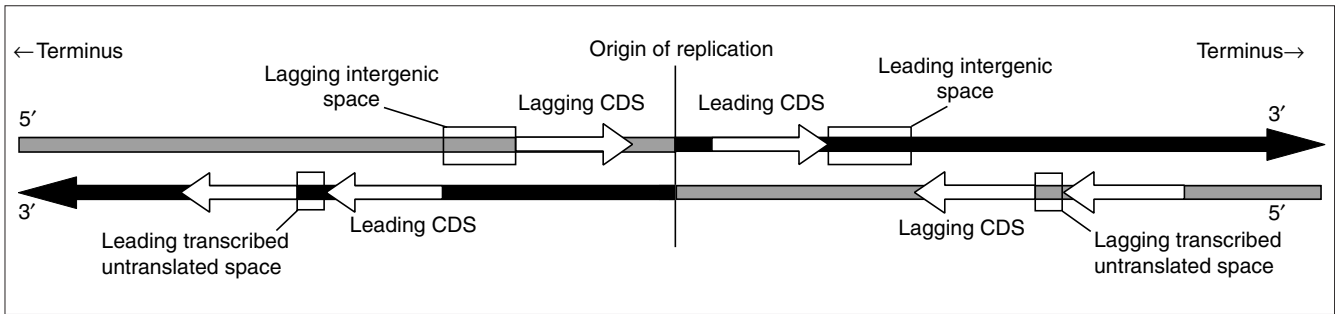


Figure 1
Representation of the strands used for analysis in this paper. The leading strand for replication is black and the lagging one is shaded gray; note the switch at the origin of replication. The sense strand of coding sequences is represented by white arrows. The sense sequence of a coding region that is transcribed in the same direction as the motion of the replication fork is in the leading strand, whereas a sense sequence that is transcribed in the opposite direction is in the lagging strand. The strand used for large intergenic spaces, represented by large boxes, is always the published strand from 5' to 3'. The strand used for potentially transcribed untranslated spaces, that is, small intergenic regions among co-oriented genes, is represented by small boxes.

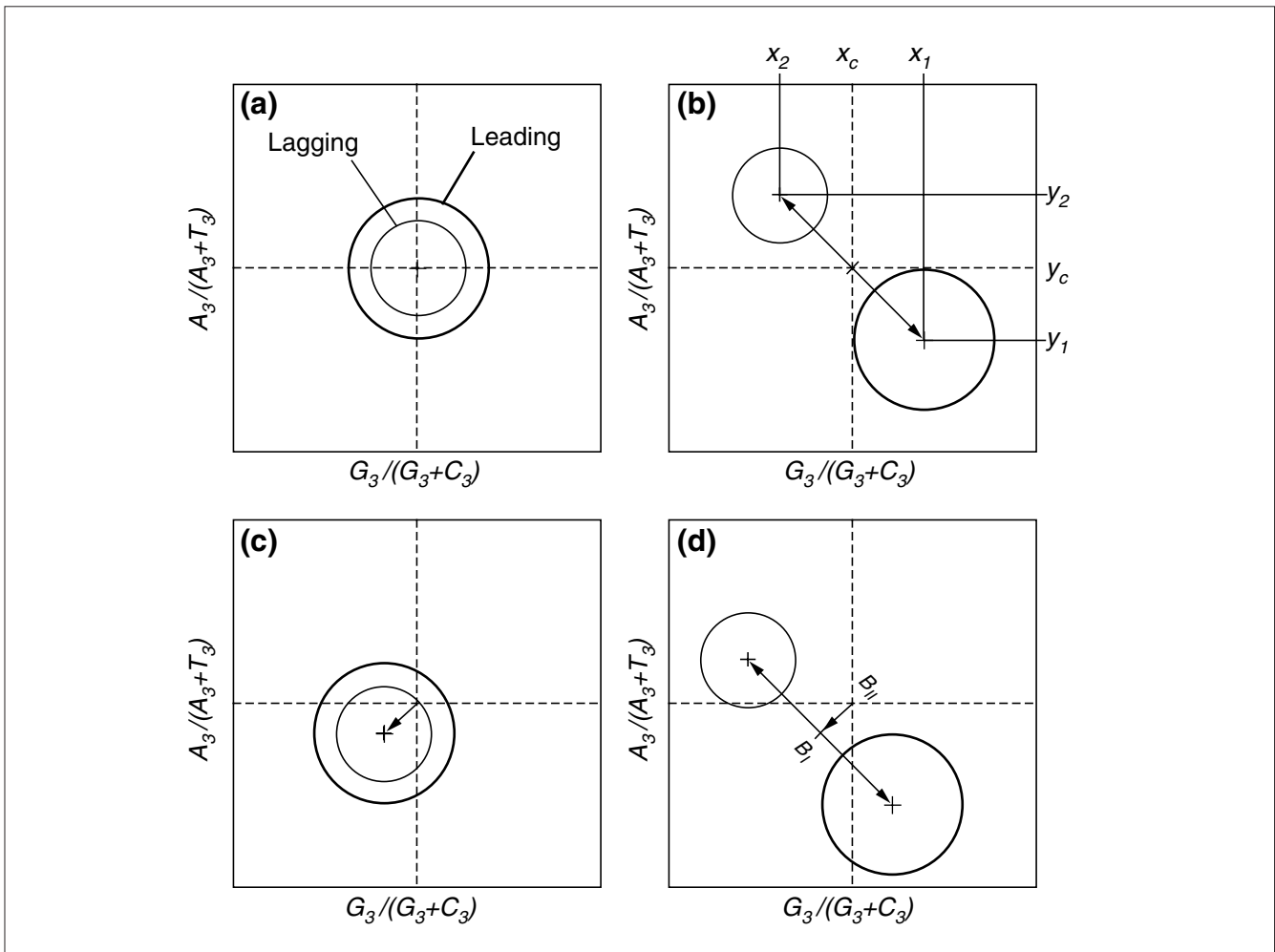


Figure 2
Expected deviations in PR2 plots in coding sequences and small intergenic regions among co-oriented genes. (a) The null hypothesis: mutation and selection are symmetric with respect to the two DNA strands; (b) replication-induced mutation pressure is asymmetric; (c) transcription- or translation-associated mutation or selection pressures are asymmetric; (d) all forces in (b) and (c) combined are asymmetric.

affected by this uneven distribution. Figure 2c shows diagrammatically what would be expected with asymmetric forces, such as codon usage that optimizes translation efficiency [49,50] or transcription-induced mutation pressure [51]. These affect the sense strand of both the leading and lagging coding sequences in the same direction, because the analyzed strand is always the sense strand. Note that the deviation from the null hypothesis could be higher [22] for the leading group because a greater number of highly expressed genes are found in this group [48], so that both transcription-induced mutation and translation-induced selection [52,53] could be higher in these genes. However, this effect was found to be negligible [35], and never produces a symmetric distribution around the center of the two groups, as in previous case (Figure 2b), because the two groups are expected to be moved in the same direction for transcription- and translation-induced biases, instead of in opposite directions for replication-induced biases. Figure 2d shows diagrammatically what would be expected when all phenomena are combined. The distance between the averages of the two groups,

$$B_I = \sqrt{(x_2 - x_1)^2 + (y_2 - y_1)^2}$$

is the contribution of replication-associated effects to PR2 deviations. The distance from the center of the plot to the midpoint of $B_I(x_c; y_c)$,

$$B_{II} = \sqrt{(x_c - 0.5)^2 + (y_c - 0.5)^2},$$

is the overall contribution of transcription- and translation-associated effects to PR2 deviations, where B_I is the replication-associated bias.

Consider now a small transcribed untranslated intergenic region between two co-oriented coding sequences. Let A, C, G, T denote its base counts in the *cis*-sense, that is in the same strand as the sense strand of the two flanking coding sequences (Figure 1). As previously described, deviations from PR2 are characterized by using $G/(G+C)$ and $A/(A+T)$ values to draw PR2-plots. Expected effects in the *cis*-sense strand are the same as in the sense strand (Figure 2) except for translation-induced selection, which is absent in untranslated regions. Therefore, a difference in B_{II} when compared with the analysis of coding sequences is evidence of translation-associated effects on PR2 deviations in coding sequences. Last, consider a large untranscribed intergenic region and note its base counts (A, C, G, T) in the published strand of the chromosome (Figure 1).

Figure 3a shows what would be expected in spacers under replication-induced effects alone when the leading strand is enriched in G and T. This is essentially the same as the situation for third codon position when there is only replication-induced mutation pressure (Figure 2b), because all bases are involved, not just those in protein-coding genes. The sole difference is that the leading and lagging group are now of similar size, because when working with the published strand, there are approximately the same number of intergenic regions in the two groups (Figure 1). Therefore, when B_I is similarly oriented in the two analyses, this is an indication of a common underlying replication-induced mutation pressure, the difference in intensity being controlled by the difference in selective pressure between the two kinds of region.

In practice, however, it is impossible to verify from DNA database annotations whether large intergenic regions are completely free from transcription. Consider an extreme theoretical case in which all large intergenic regions are

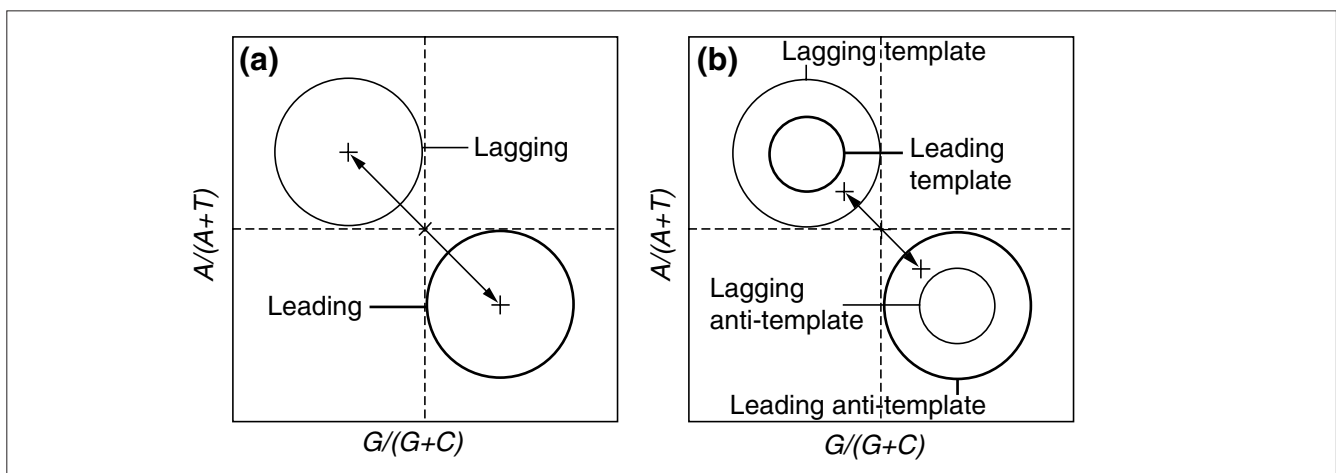


Figure 3

Expected deviations in PR2 plots in large intergenic spaces **(a)** when replication-induced mutation pressure is asymmetric and **(b)** in an extreme case of asymmetric transcription-induced forces (see text for details).

transcribed (Figure 3b). For simplicity, the whole intergenic region is assumed to be transcribed from the same strand: the whole intergenic region is either the template strand or the anti-template strand, but not a mixture of both. (The anti-template strand has the same nucleotide sequence as the transcribed RNA because the complementary strand is the template strand for transcription.) This simplification does not alter the reasoning, because a proper partition of intergenic regions would remove the mixed cases. In the leading-strand group, positioned to the right of the origin (Figure 1), there is an excess of anti-template strand for transcription because of the selection against head-on collisions between RNA and DNA polymerase complexes [54-56]. Therefore, the anti-template strand subgroup is bigger than the template one in the leading group, and by a symmetrical reasoning, on the left to the origin, the template strand subgroup is bigger than the anti-template one in the lagging-strand group. Figure 3b shows what is expected if transcription leads to an excess of G and T bases in the anti-template strand. Note that in this extreme case transcription effects yield $B_I \neq 0$, but the general pattern (Figure 3b) is completely different from replication-induced mutation pressure effects (Figure 2b), making it easy to discriminate between the two effects.

Results

Distribution of G+C content

Thanks to the availability of an increasing number of complete bacterial genomes with a high G+C content, there was a representative distribution of G+C content in the dataset (Table 1), and the values of G+C content in third codon positions (P_3 , as previously defined in [11]; see also Materials and methods), in first and second codon positions averaged (P_{12}), and in intergenic spaces (GC_{IGR}), were found to be consistent with previously known results [10,11] both in terms of their range of distribution and the correlation between them (Figure 4). Intragenomic P_3 distributions of all species examined (all histograms are given in the tables in Additional data files under the 'GCOrder' flag) are unimodal and narrow for a major class of genes comprising more than 90% of the total genes, although average P_3 values differ widely between species. Some species show scattering of P_3 distribution. In particular, a previous study of 254 genes in *Pseudomonas* [7] revealed a sizeable minor class of genes (28%) forming a trail toward lower G+C content (genes with $P_3 < 0.75$). In our present study of 5,255 genes, the minor class of genes (with $P_3 < 0.75$) is only 8% of the total, indicating that the previous sample was biased towards the minor class. Moreover, the width of the distribution (standard deviation) of the majority of genes is narrower in species with both extreme ranges of P_3 than in those with middle ranges. These features of the P_3 distribution are expected from the theory of bidirectional mutation rates and their equilibrium [8]. In this theory, mutation rates, u ($GC \rightarrow AT$) and v ($AT \rightarrow GC$), will lead to a G+C

content (P_3) at equilibrium of $v/(u+v)$. The expected standard deviation is

$$\sqrt{P_3(1-P_3)/b}$$

where b is an average of the number of third codon positions per gene [8].

Difference in G+C content between leading and lagging strands

Statistically highly significant differences ($p < 0.01$) in P_3 between coding sequences located on the leading and lagging strands were found in 23 out of 43 chromosomes. In most cases (21 out of the 23) genes on the leading strand were found to have a lower P_3 content than those on the lagging one. (Two exceptions were *Mycoplasma genitalium*, which is known to have a peculiar behavior with respect to G+C content [5,6], and *Caulobacter crescentus*.) As far as we know, this is the first time that such a systematic difference has been reported, but note that the differences were always very small. The maximum was only 0.035, for the difference in P_3 content of leading and lagging strands of *Vibrio cholerae* chromosome 2, as compared to the 0.768 average P_3 difference between *Halobacterium* sp. and *Ureaplasma urealyticum*, or the 0.050 accepted range of genomic G+C content polymorphism [57]. There was no significant correlation between the average P_3 and the difference in P_3 between the two groups of genes. A highly significant difference in G+C content between the leading and the lagging sequences was found in only five chromosomes for large intergenic regions, and three chromosomes for small intergenic spaces.

PR2-biases in coding sequences

Out of 43 chromosomes, the differences in PR2-biases between the leading and lagging groups were highly significant ($p < 0.01$) in 39 chromosomes for $G_3/(G_3+C_3)$ and in 34 for $A_3/(A_3+T_3)$ and in 32 chromosomes for simultaneously $G_3/(G_3+C_3)$ and $A_3/(A_3+T_3)$ (see Additional data files). In the PR2-bias-plot analysis, we found a general pattern of $x_1 > x_2$ and $y_1 < y_2$, as in Figure 2b, meaning that coding sequences in the leading strand had higher $G_3/(G_3+C_3)$ values and lower $A_3/(A_3+T_3)$ values than those on the lagging strand: leading coding sequences were enriched in keto-bases (G and T) in the third codon position compared to lagging coding sequences. There were, however, exceptions to this general trend: out of the 39 chromosomes that were highly significant for $G_3/(G_3+C_3)$, all had higher values in the leading strand, but out of the 34 chromosomes that were highly significant for $A_3/(A_3+T_3)$, three chromosomes did not follow the general trend (*Lactococcus lactis* and *Staphylococcus aureus* strains Mu50 and N315). Leading and lagging coding sequences are separated in PR2 plots as expected under replication-associated effects, and the leading group is almost always down right of the lagging group. However, the extent to which the

Table 1**List of 51 chromosomes under study**

Chromosome	Strain	ACC	EMGLib	Genome (bp)	Chir	G+C (%)	P_{12}	P_3	GC_{IGR}
<i>Aeropyrum pernix</i>	K1	AP0000 [58-64]	CG0043	1,669,695	No	56.3	0.567	0.662	0.506
<i>Aquifex aeolicus</i>	VF5	AE000657	CG0034	1,551,335	No	43.5	0.442	0.488	0.386
<i>Archaeoglobus fulgidus</i>	DSM 4304	AE000782	CG0032	2,178,400	No	48.6	0.507	0.489	0.377
<i>Bacillus halodurans</i>	C-125	BA000004	CG0057	4,202,353	Yes	43.7	0.466	0.403	0.384
<i>Bacillus subtilis</i>	168	AL009126	CG0031	4,214,814	Yes	43.5	0.455	0.424	0.364
<i>Borrelia burgdorferi</i>	B31	AE000783	CG0033	910,724	Yes	28.6	0.347	0.197	0.212
<i>Buchnera</i> sp.	APS	AP000398	CG0058	640,681	Yes	26.3	0.360	0.122	0.155
<i>Caulobacter crescentus</i>	CB15	AE005673	CG0068	4,016,947	Yes	67.2	0.600	0.856	0.625
<i>Campylobacter jejuni</i>	NCTC11168	AL111168	CG0047	1,641,481	Yes	30.6	0.381	0.175	0.205
<i>Chlamydia muridarum</i>	MoPn	AE002160	CG0054	1,069,412	Yes	40.3	0.462	0.317	0.360
<i>Chlamydia trachomatis</i>	D/U W-3/Cx	AE001273	CG0037	1,042,519	Yes	41.3	0.468	0.330	0.360
<i>Chlamydophila pneumoniae</i>	CWL029	AE001363	CG0041	1,230,230	Yes	40.6	0.459	0.332	0.323
<i>Chlamydophila pneumoniae</i>	AR39	AE002161	CG0053	1,229,853	Yes	40.6	0.460	0.271	0.340
<i>Chlamydophila pneumoniae</i>	J138	BA000008	CG0062	1,228,267	Yes	40.6	0.459	0.332	0.323
<i>Deinococcus radiodurans</i> chromosome 1	R1	AE000513	CG0049	2,648,638	Yes	67.0	0.622	0.801	0.623
<i>Deinococcus radiodurans</i> chromosome 2	R1	AE001825	CG0050	412,348	Yes	66.7	0.605	0.824	0.593
<i>Escherichia coli</i>	K-12	U00096	CG0028	4,639,221	Yes	50.8	0.514	0.534	0.425
<i>Escherichia coli</i>	EDL933	AE005174	CG0069	5,528,970	Yes	50.4	0.513	0.527	0.425
<i>Escherichia coli</i>	RIMD 0509952	BA000007	CG0070	5,498,450	Yes	50.5	0.514	0.528	0.424
<i>Halobacterium</i> sp.	NRC-1	AE004437	CG0065	2,014,239	No	67.9	0.600	0.880	0.634
<i>Haemophilus influenzae</i>	KW20	L42023	CG0001	1,830,140	Yes	38.2	0.447	0.265	0.312
<i>Helicobacter pylori</i>	26695	AE000511	CG0001	1,667,877	Yes	38.2	0.396	0.404	0.302
<i>Helicobacter pylori</i>	J99	AE001439	CG0042	1,643,831	Yes	39.2	0.397	0.411	0.308
<i>Lactococcus lactis</i>	IL1403	AE005176	CG0074	2,365,589	Yes	35.5	0.425	0.229	0.276
<i>Methanobacterium</i> <i>thermoautotrophicum</i>	H	AE000666	CG0030	1,751,377	No	49.5	0.525	0.500	0.383
<i>Methanococcus jannaschii</i>	DSM 2661	L77117	CG0003	1,664,977	No	31.4	0.347	0.307	0.252
<i>Mycobacterium leprae</i>	TN	AL450380	CG0071	3,268,203	Yes	57.8	0.576	0.638	0.546
<i>Mycobacterium tuberculosis</i>	H37Rv	AL123456	CG0035	4,411,529	Yes	65.6	0.604	0.787	0.625
<i>Mycobacterium tuberculosis</i>	CDC1551	AE000516	CG0073	4,403,836	Yes	65.6	0.605	0.783	0.627
<i>Mycoplasma genitalium</i>	G-37	L43967	CG0002	580,073	Yes	31.7	0.367	0.221	0.272
<i>Mycoplasma pneumoniae</i>	M129	U00089	CG0011	816,394	Yes	40.0	0.409	0.403	0.339
<i>Mycoplasma pulmonis</i>	UAB CTIP	AL445566	CG0072	963,879	Yes	26.6	0.343	0.140	0.182
<i>Neisseria meningitidis</i>	Z2491 (A)	AL162759	CG0056	2,184,406	Yes	51.8	0.503	0.598	0.444
<i>Neisseria meningitidis</i>	MC58 (B)	AE002098	CG0055	2,272,351	Yes	51.5	0.502	0.593	0.447
<i>Pasteurella multocida</i>	PM70	AE004439	CG0075	2,257,487	Yes	40.4	0.453	0.323	0.329
<i>Pseudomonas aeruginosa</i>	PA01	AE004091	CG0059	6,264,403	Yes	66.6	0.583	0.870	0.616
<i>Pyrococcus abyssi</i>	GE5	AL096836	CG0045	1,765,118	Yes	44.7	0.457	0.511	0.379
<i>Pyrococcus horikoshii</i>	OT3	AP00000 [1-7]	CG0038	1,738,505	Yes	41.9	0.449	0.428	0.376
<i>Rickettsia prowazekii</i>	Madrid E	AJ235269	CG0040	1,111,523	Yes	29.0	0.387	0.168	0.242
<i>Staphylococcus aureus</i>	Mu50	BA000017	CG0076	2,878,134	Yes	32.9	0.404	0.205	0.277
<i>Staphylococcus aureus</i>	N315	BA000018	CG0077	2,813,641	Yes	32.8	0.404	0.204	0.274
<i>Streptococcus pyogenes</i>	SF370	AE004092	CG0078	1,852,441	Yes	38.5	0.439	0.297	0.325

Table 1 (continued)

Chromosome	Strain	ACC	EMGLib	Genome (bp)	Chir	G+C (%)	P_{12}	P_3	GC_{IGR}
<i>Sulfolobus solfataricus</i>	P2	AE006641	CG0079	2,992,245	No	35.8	0.409	0.325	0.310
<i>Synechocystis</i> sp.	PCC 6803	AB001339	CG0010	3,573,470	No	47.7	0.503	0.468	0.421
<i>Thermoplasma acidophilum</i>	DSM 1728	AL139299	CG0060	1,564,906	Yes	46.0	0.473	0.558	0.361
<i>Thermotoga maritima</i>	MSB8	AE000512	CG0044	1,860,725	Yes	46.2	0.456	0.524	0.397
<i>Treponema pallidum</i>	Nichols	AE000520	CG0036	1,138,011	Yes	52.8	0.537	0.538	0.541
<i>Ureaplasma urealyticum</i>	ATCC 700970	AF222894	CG0048	751,719	Yes	25.5	0.334	0.112	0.178
<i>Vibrio cholerae</i> chromosome 1	N16961	AE003852	CG0063	2,961,149	Yes	47.7	0.494	0.470	0.412
<i>Vibrio cholerae</i> chromosome 2	N16961	AE003853	CG0064	1,072,315	Yes	46.9	0.482	0.458	0.431
<i>Xylella fastidiosa</i>	9a5c	AE003849	CG0061	2,679,306	Yes	52.7	0.535	0.552	0.468

The first column is the species names with chromosome number when necessary, the second the strain that was sequenced. ACC is the accession number in the DDBJ/EMBL/GenBank database, EMGLib the accession number in EMGLib database [88]. Chir denotes whether a clear chirochore structure allowed for the classification of subsequences (for example, CDS) in the leading or lagging group. The G+C contents are given for the whole genome (G+C), in first and second codon positions (P_{12}), in third codon positions (P_3), and large intergenic spaces (GC_{IGR}) as defined in the Materials and methods section.

groups are separated differs between species. The two most extreme species are *B. burgdorferi*, where the two groups of genes are completely resolved, and *M. genitalium*, where the two groups are almost completely overlapping (Figure 5). Other species with a spectacular difference in PR2 plots were *Chlamydia muridarum*, *Chlamydia trachomatis* and *Treponema pallidum*. Out of 43 chromosomes, the residual bias B_{II} was in most cases oriented as in Figure 2c, with $x_c < 0.5$ (all except *Pyrococcus abyssi* and *T. pallidum*) and $y_c < 0.5$ (all except *Thermotoga maritima*). The relative contribution of replication-coupled bias (B_I) to the total bias (B_I plus B_{II}) ranged from 6% (*Mycoplasma pulmonis*) to 86% (*B. burgdorferi*) with an average value of 52%.

PR2-biases in large intergenic regions

Out of 43 chromosomes, the differences in PR2-biases between the leading and lagging groups were highly significant ($p < 0.01$) in 36 chromosomes for G/(G+C) and in 34 for A/(A+T) and in 32 chromosomes for simultaneously G/(G+C) and A/(A+T) (see table in Additional data files). All the 36 chromosomes highly significant for G/(G+C) had higher values in the leading strand (that is, $x_1 > x_2$ as in coding sequences). Out of the 34 chromosomes highly significant for A/(A+T), 28 had lower values in the leading strand (that is, $y_1 < y_2$ as in coding sequences). Exceptions were *Bacillus subtilis*, *Bacillus halodurans*, *L. lactis*, *S. aureus* strains Mu50 and N315, and *Streptococcus pyogenes*. The distribution of intergenic regions in a PR2-plot was as expected under replication-associated effects (Figure 3a), and is particularly visible in the case of *B. burgdorferi* (Figure 5c). Unlike the third codon position, the leading and lagging groups clustered symmetrically around the center point of the PR2-bias plot. In intergenic regions, both x_c and y_c were always near 0.5, so that the residual bias B_{II} was always close to zero. The relative contribution of replication-coupled bias (B_I) to the total bias (B_I plus B_{II}) was therefore

very high, on average 90%, and ranged from 88% (*Helicobacter pylori*) to 99.6% (*B. subtilis*) in the intergenic regions of species with significant B_I values.

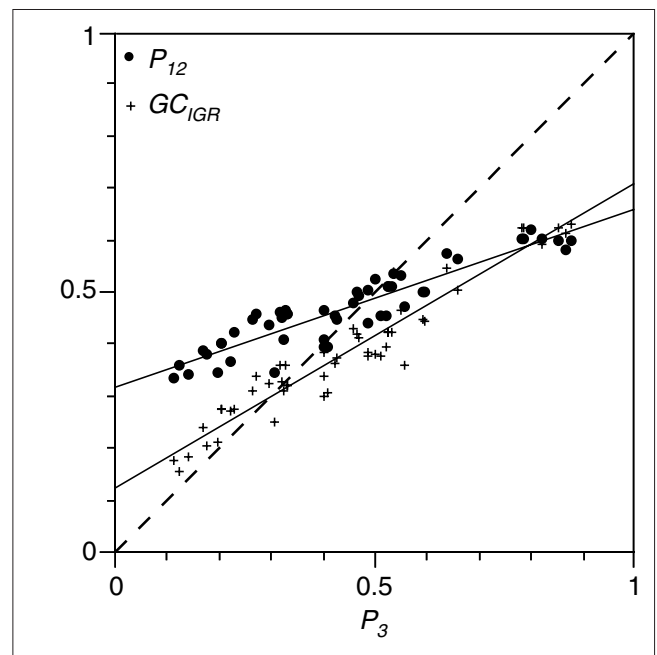


Figure 4
Distribution of G+C content in the dataset. The G+C content of the 51 bacterial chromosomes under analysis was highly variable from 25.5% in the *Ureaplasma urealyticum* chromosome to 67.9% in *Halobacterium* sp., with a larger distribution in third codon positions (x-axis P_3 from 11.2% to 88.0%) than in intergenic spaces (y-axis GC_{IGR} from 15.5% to 63.4%) than in first and second position (y-axis P_{12} from 33.4% to 62.2%) as expected [10,11]. Regression slopes (or ϵ values) and their standard deviations for P_{12} and GC_{IGR} were 0.343 ± 0.021 and 0.586 ± 0.024 , respectively.

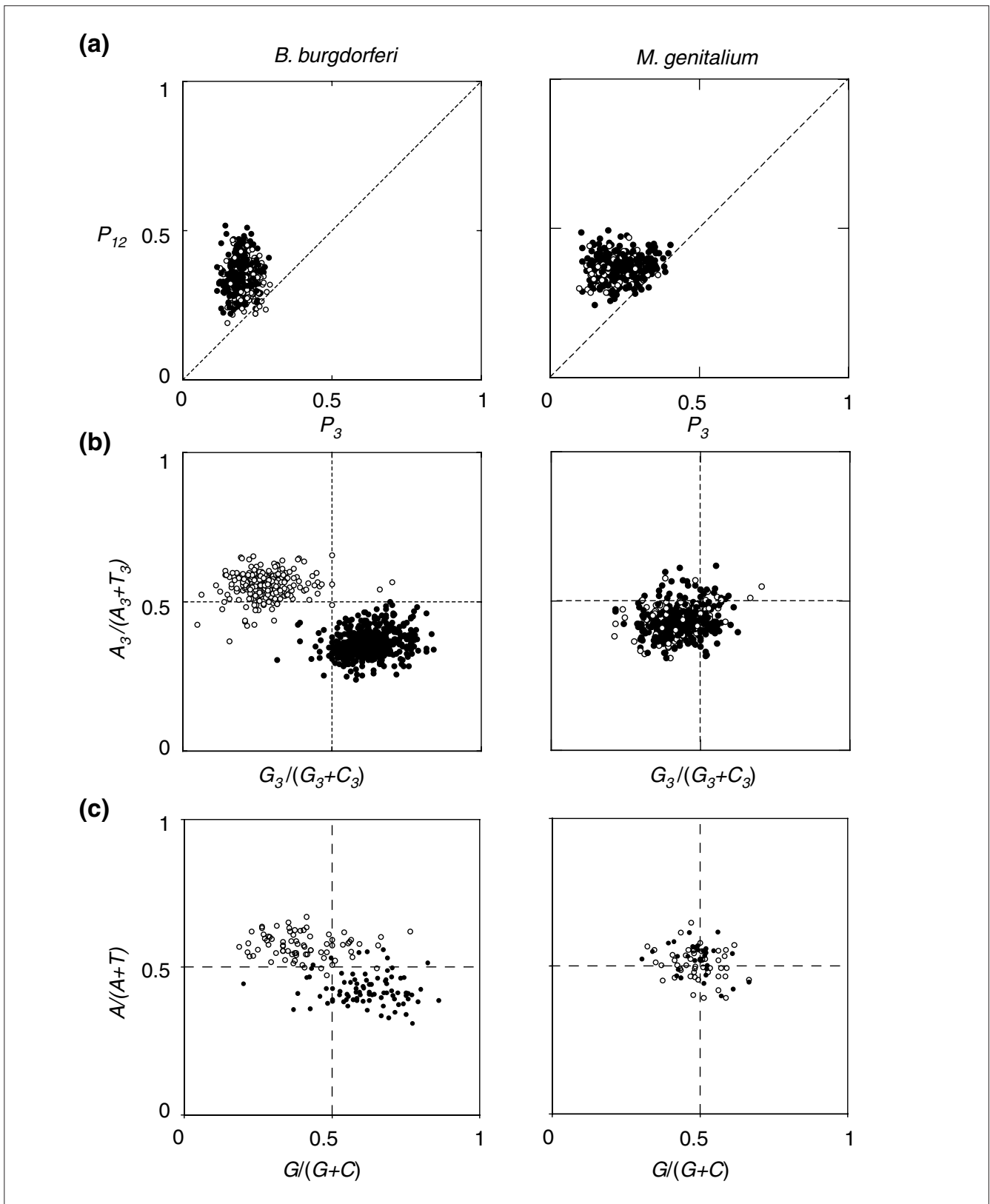


Figure 5
 Example of detailed results for two extreme cases with *B. burgdorferi* (left) and *M. genitalium* (right). **(a)** P_3 - P_{12} plot for coding sequences; **(b)** PR2-plot in third codon position; **(c)** PR2 plot in large intergenic spaces. Leading-group sequences are represented by black circles, and lagging ones by white circles.

PR2-biases in potentially transcribed untranslated regions

Out of 43 chromosomes, the differences in PR2-biases between the leading and lagging small intergenic spaces among co-oriented genes were highly significant ($p < 0.01$) in 26 chromosomes for G/(G+C) and in 7 chromosomes for A/(A+T) and in 7 chromosomes for both G/(G+C) and A/(A+T) together (see table in Additional data files). All the 26 chromosomes with highly significant difference in G/(G+C) showed the same pattern as in third codon positions in coding sequences with $x_1 > x_2$. Of the seven chromosomes highly significant for A/(A+T), all showed the same pattern as in third codon positions in coding sequences with $y_1 < y_2$, except for *B. subtilis* and *S. pyogenes*. Out of 43 chromosomes, the orientation of the residual bias B_{II} showed no clear tendency (only 31 chromosomes had $x_c > 0.5$ and 23 $y_c > 0.5$; the trend, if any, would be in the opposite direction, as in third codon positions). The relative contribution of replication-coupled bias (B_I) to the total bias (B_I plus B_{II}) ranged from 10% (*H. pylori* strain 26695) to 93% (*Thermoplasma acidophilum*) with an average value of 58%.

Comparison of third codon positions and intergenic spaces

The differences between PR2-biases in intergenic regions between the leading and lagging strands (B_I) were significantly and highly correlated with the PR2-biases in the third codon position as is evident from the regression coefficient of approximately 0.6 and correlation coefficient ($r^2 = 0.77$) among 43 chromosomes (Figure 6). The correlation was still

significant when the extreme *B. burgdorferi* was removed from analysis ($r^2 = 0.68$). The 95% confidence interval for the value of the slope of the regression line [0.50-0.71] is less than 1, meaning that replication-associated biases were significantly smaller in intergenic regions than in third codon positions. This slope value was very close to the one obtained for the regression line between G+C content in intergenic regions versus third codon positions (Figure 4).

Correlation between P_3 and replication-associated biases

If differences in the G+C content between species were dictated by asymmetric replication-associated mutation pressure, one would expect a correlation between the extent of PR2-bias and G+C content; however, no significant correlation between B_I in coding sequences and P_3 were found (Figure 7).

Discussion
Asymmetric mutation pressure

A general feature observed in the present study is that, whenever there is a difference in base composition between the leading and the lagging strand, the G and T bases are enriched in the leading strand, and the G+C content is somewhat lower in the leading strand (see Results). The direction of observed biases are universal in bacteria [28], although not every species is biased [58]. This pattern is not restricted to the bacterial world, as GT-enriched leading strands have been documented in mitochondria [59-61], in the chloroplast

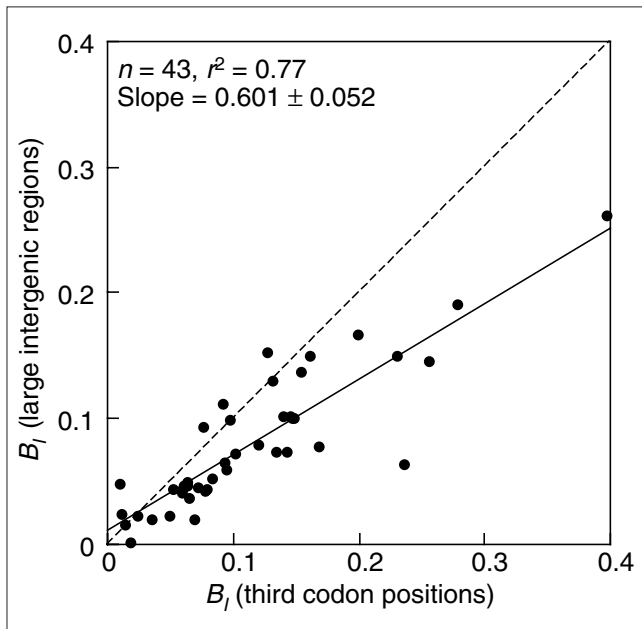


Figure 6
Comparison of the absolute contribution of replication-associated bias B_I between intergenic and third codon positions.

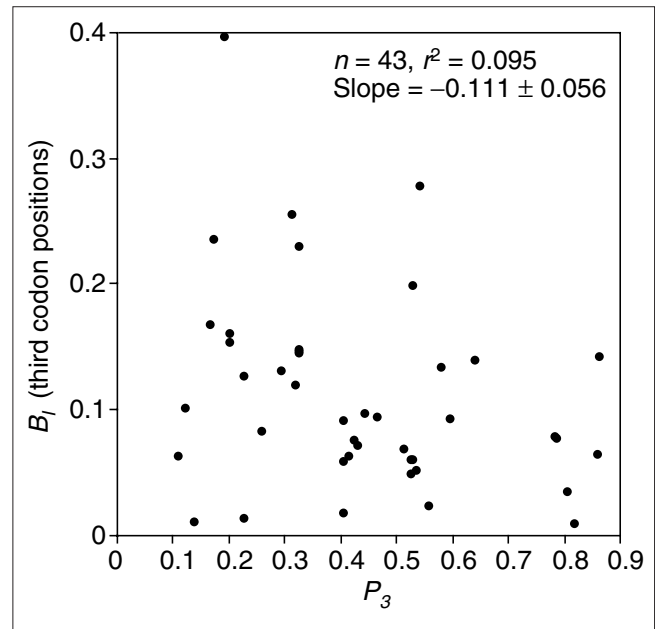


Figure 7
Correlation between GC content and the extent of strand biases in third codon positions.

genome of *Euglena gracilis* [62], in viruses [45-47], and in the telomeric ends of the *Saccharomyces cerevisiae* genome [63]. Not all sequences are biased, as shown by the well-documented β -globin region in the *Homo sapiens* genome [37]. Remarkably, absolutely no exceptions to the relative enrichment in G of the leading strand were found here, either in third codon positions or intergenic positions. However, the sole general rule in biology is that there are no general rules, and during the time of revision of this manuscript the first exception was documented in the chromosome of *Streptomyces coelicolor* [64]. The relative enrichment in T of the leading strand suffers from some exceptions that, interestingly, are all found here in the low-G+C group of Gram-positive bacteria (namely *B. subtilis*, *B. halodurans*, *L. lactis*, *S. aureus*, and *S. pyogenes*).

Because of the strong correlation in direction and intensity of PR2 deviation between third codon positions and intergenic spaces (Figure 6), the most likely explanation for the GT enrichment of the leading strand is a replication-induced asymmetric mutation pressure between the leading and lagging strand.

Potential underlying causes

The universal excess of G and T in the leading strand is common to distantly related species, suggesting that asymmetric directional mutation pressure has a chemical basis. This does not mean that the mechanism is simple: there are 12 substitution rates under asymmetric molecular evolution, but only four base frequencies; many different substitution scenarios can explain the observed biases. Here we discuss only simple hypotheses in which only one of the 12 substitution rates is assumed to differ between the two strands: for example, only an excess of $A \rightarrow G$ or of $C \rightarrow T$ in the leading strand is able to produce a GT-rich leading strand. Because the leading strand spends more time in the single-stranded state than the lagging strand during DNA replication [65], it is natural to look for mutations that are increased in the single-stranded state as possible explanations.

Oxidations

We are not aware of any studies comparing the rate of base oxidation between the single-stranded and double-stranded state in DNA. However, in a study of peroxy radicals [66], which have a longer biological half-life than other O_2 -derived oxidants, all products formed were from the major groove of the bases, implying that steric hindrance from Watson-Crick base pairing in double-stranded DNA is not a factor. Consequently, similar yields of products in both single- and double-stranded DNA were found. Additionally, 8-hydroxyguanine, a major product of oxidative damage in DNA, induces mainly $G \rightarrow T$ and $A \rightarrow C$ mutations [67,68]. In *Escherichia coli*, it has also been reported that 2-hydroxydATP induces $G \rightarrow T$ mutations [69]. These results do not explain GT excess in the leading strand and therefore are not

good candidates for a single mechanism of asymmetric mutation pressures.

Deaminations

Within this group, only cytosine deamination to uracil (or 5-methylcytosine deamination directly to thymine) yields a $C \rightarrow T$ mutation, and only adenine deamination to hypoxanthine yields an $A \rightarrow G$ mutation. Interestingly, there is experimental evidence *in vitro* that the rate of these deaminations is increased in the single-stranded state. Deamination of cytosine occurs over 100 times faster in single-stranded than in double-stranded DNA [70,71]. This is also most likely true for adenine, but its deamination rate is one-fortieth of the rate of cytosine [72], so that it is not possible to determine its rate in double-stranded DNA. The rate-determining step in cytosine deamination involves attack by water at the C-4 of the cytosine to give a tetrahedral intermediate, difficult to obtain in the rigid structure of double-stranded DNA because of Watson-Crick base pairing with guanine [73]. Single-stranded-DNA-binding proteins do not offer specific protection from this type of mutation during DNA replication [74] but may differ between species in their ability to protect from solvent attack. The single-stranded state during transcription induces $C \rightarrow T$ mutations [75], and this effect increases with transcription level [76] or when the duration of the single-stranded state is increased using a slower, mutant, RNA polymerase [77]. Also consistent is the report [78] that GT enrichment increases with the time spent in the single-stranded state during mitochondrial DNA replication. In a study of the mutation pattern estimated from pseudogene data in *B. burgdorferi*, it was found that $C \rightarrow T$ mutation was the most frequent in the leading strand [79]. The small depletion in G+C content observed here in the leading strand is also consistent with an excess of $C \rightarrow T$ substitutions in the leading strand because $C \rightarrow T$ substitutions decrease the G+C content. This effect is also expected to be a second-order one because of the centripetal action of symmetric directional mutation pressure on the G+C content of both strands. Therefore, we found the cytosine deamination theory compatible in most cases with the data, but a more definitive answer may come from biochemical studies on an extreme case such as *B. burgdorferi*.

G+C content variation between species is not controlled by a unique asymmetric mutation pressure

From inspection of our data, we can reject the simple model in which a unique asymmetric mutation pressure causes variation in G+C content between species. For instance, if only $C \rightarrow T$ mutations were the underlying phenomenon, we would expect low G+C in species with the highest B_L . Figure 7 shows that this is clearly not the case. Moreover, if asymmetric directional mutation pressures were the major origin of variation in G+C content between species, we would expect a much more important difference in term of G+C content between the genes on the leading strand and those on the lagging strand.

Intergenic regions could be under greater selective constraints than expected

If we exclude as an explanation that many unannotated genes remain to be discovered, or that the boundaries of annotated genes should be greatly extended, so that some intergenic regions under analysis are not actually intergenic, then, there are two non-exclusive interpretations of the fact that B_I is smaller in intergenic regions than in third codon positions. On the one hand, one may postulate that intergenic regions are under weaker selection pressure against inversions, so that deviations from PR2 are cancelled. On the other hand, one may assume that some positions are not free to deviate from PR2 because of selective pressure for some function. For instance, there are 100 copies per enterobacterial genome of palindromic sequences in intergenic spaces [80], which may be under selective pressure to preserve their palindromic character and therefore follow PR2 (as pure palindromic sequences are effectively base-paired). This interpretation is compatible with the fact that interspecific G+C content variation is higher at the third codon position than in intergenic spaces [10,11], as shown here in Figure 4. Moreover, the surprisingly low level of polymorphism in large intergenic regions among natural strains of *E. coli* [81] is in favor of selective constraints.

Transcription-coupled PR2-biases

Francino and Ochman [22,81] pointed out that transcription is likely to cause more mutations in the sense strand than in the anti-sense strand because of its transient single-stranded state during transcription. We found no evidence for this in intergenic spaces (> 100 bp), which could be interpreted either as an absence of transcription-induced mutation pressure or by a low proportion of transcribed fragments in these regions. Third codon positions are known to be transcribed, so that we expect an effect of transcription-induced mutation pressure on the residual deviation from PR2 (B_{II}). However, selection for translation optimization interferes, so that we cannot interpret unambiguously the almost universal orientation of B_{II} (down left in PR2-plot, as in Figure 2c) as the result of transcription-induced mutation pressure. If we interpret results this way, we would have to postulate a different mechanism for replication-induced and transcription-induced mutation pressures, as B_I and B_{II} have different orientations. Moreover, in small intergenic spaces, which are potentially transcribed untranslated regions, no general pattern is evident for the residual deviation from PR2, so that no universal transcription-induced mutation pressure was found here as a strong contributor to the deviation from the PR2 state.

Asymmetric mutation pressure and chirochore structure

A chirochore is a contiguous segment of the chromosome homogeneous with respect to its deviation from PR2 [44]. A strong asymmetric mutation pressure induces a chirochore structure, as observed in *B. burgdorferi* [82], but the

converse need not to be true: a weak asymmetric mutation pressure does not imply an absence of chirochore. For example in *M. genitalium*, replication-associated biases, $B_I/(B_I + B_{II})$, are the weakest among species under study ($B_I = 12\%$), but there is still a strong chirochore structure [83] because of a large excess (80%) of coding sequences in the leading strand. The deviation from PR2 in the first and second codon positions, because of selection at the amino-acid level (for example [84]), is then a major factor. It is not known whether chirochores could be adaptative, but recent results suggest a functional polarization of the *E. coli* chromosome [85,86] and may be relevant to this problem.

PR2-plot analysis

A prerequisite for using the simple graphical representations and quantifications used here is that the coding sequences and intergenic spaces can be divided into two groups: the leading and the lagging group. This is not necessarily always possible when, for instance, all coding sequences are in the leading group (this situation has never been encountered in bacteria but could be problematic in small genomes such as those of mitochondria). Another limitation is encountered when there are genes in both groups but we are unable to assign sequences to the correct group because of limited information about the location of origin and terminus of replication used during the course of evolution. Although an estimation of B_I is not possible in this latter case, an oblong distribution of points in PR2-plots may suggest a non-zero value for B_I . When more than one strain was sequenced for a species (that is, *Chlamydomonas reinhardtii*, *E. coli*, *H. pylori*, *Mycobacterium tuberculosis*, *Neisseria meningitidis*, and *S. aureus*) B_I and B_{II} values in third codon position are extremely stable within a species (see table in the Additional data files), but the maximum number of three strains available here for one species does not allow us to estimate their within-species variability, and therefore their taxonomic utility.

Materials and methods

Source of data

Data on 51 complete bacterial chromosomes were retrieved from the EMGLib server [87,88] and used to extract annotated coding sequences and intergenic spaces (Figure 1). These 51 chromosomes were from 49 different strains (there are two chromosomes in *Deinococcus radiodurans* and *Vibrio cholerae*), from 41 different species (there were three strains of *C. pneumoniae*, *E. coli*; two strains of *H. pylori*, *M. tuberculosis*, *N. meningitidis*, *S. aureus*) as detailed in Table 1.

Leading and lagging group assignment

To classify sequences into the leading and the lagging groups (Figure 1), we must be able to locate them relative to the origin and terminus of replication. Out of 51 bacterial chromosomes, the locations of the origin and the terminus were

known from experimental evidence or were estimated from chirochore boundaries [44] in 43 chromosomes with the program Oriloc [89,90]. Details of assignments are given in EMGLib database under a '/strand=' qualifier. To discriminate between the origin and the terminus of replication, we used the assumption that organisms tend to avoid head-on collisions between the DNA replication apparatus and the RNA polymerase transcription complex [54-56]. We also assumed that coding sequences were more abundant on the leading than on the lagging strand. As far as we know, there is no exception to this rule, and the report that the trend is exacerbated in highly expressed genes supports this assumption [48]. Moreover, the comparison of the maps of closely related bacterial chromosomes shows that observed chromosome rearrangements tend to preserve an excess of genes in the leading strand [91-98]. Our figures show sequences on the leading strand as filled circles, and those on the lagging strand as open circles.

Coding-sequence analysis

The nucleotide composition of coding sequences was always calculated from the sense strand (Figure 1) for each codon position. The sense strand (or anti-template strand) is the DNA strand opposite to the template strand for transcription. The average G+C content of the third codon position (P_3) and that of the first and second codon positions (P_{12}) were computed to draw neutrality plots relative to P_3 as previously described [11]: stop codons and codons from odd-numbered sets ATG (Met), TGG (Trp) and ATA (Ile) were excluded from the calculations of P -values below to avoid an extra cause of potential deviation from PR2. The coding sequences after position 1,950 kb on *D. radiodurans* chromosome 1 were discarded because of a frameshift error in the annotations. Some of the apparent coding sequences identified in genome sequences could be artifacts that do not correspond to actually expressible genes [99]. To overcome this problem, coding sequences of less than 300 bp were removed from the analysis (less than 10% of sequences were discarded). As the probability of obtaining an open reading frame with more than 300 bp by chance is low, most (> 99%) sequences analyzed should correspond to actual coding sequences [100].

Intergenic-sequence analysis

G_{IGR} denotes the G+C content, and deviations from PR2 were characterized by $G/(G+C)$ and $A/(A+T)$ to draw PR2-plots in a way similar to coding-sequence analyses. Intergenic sequences were split into two non-overlapping groups (Figure 1). The first group contained small intergenic sequences among co-oriented genes that are potentially transcribed untranslated spacers. The strand used to compute nucleotide composition - chosen to allow direct comparisons with coding-sequence analyses - is the same as the anti-template strand for transcription of the two flanking genes (CDS, tRNA, rRNA). Therefore, when the two flanking genes are coding sequences, the strand is the same as the

strand used for the analysis of coding sequences, that is the sense strand (Figure 1). Their minimum size was set to 50 bp, a trade-off between the likelihood of being transcribed and the avoidance of too much scattering in PR2-plots. Their maximum size was set to 100 bp to avoid data overlap with large intergenic regions. The second group contained large intergenic regions that are potentially untranscribed untranslated regions. Their minimum size was set to 100 bp, which is an amount of information equivalent to the third codon position of 100 codons, the minimum size required in coding-sequence analyses. The analyzed strand cannot be defined unambiguously with respect to the flanking genes when they are convergently or divergently transcribed. The nucleotide composition was therefore always calculated from the published strand (Figure 1).

Statistical tests and their interpretation

The significance of the analyses was assessed using unpaired t -test implemented in Statview 5.0 (SAS Institute Inc., North Carolina, USA) to compare the mean between two groups - here the leading and the lagging group. The null hypothesis is the equality of the mean value between the two groups. The critical value p is the probability of taking the wrong decision of rejecting the null hypothesis when the null hypothesis is true. Two notes of caution should be introduced about the interpretation of results in the present context. First, we made runs of 43 comparisons so that the odds of getting at least one wrong significant result, $1 - (1 - p)^{43}$, is 0.89, 0.35, 0.04 for $p = 0.05$, $p = 0.01$, $p = 0.001$ respectively. We selected the highly significant critical level ($p < 0.01$) so that odds of getting one, two, or three wrong decisions were 0.28, 0.06, and 0.01, respectively, for each run. Therefore, a simple figure to keep in mind when looking at the results is that the odds of getting by chance three or more highly significant differences in a run were less than 1 in 100. The second note of caution is that we were working with large data sets, typically one thousand values within each group, so that the power of the test, that is its ability to reject the null hypothesis, was high. As a matter of consequence, even a small difference between the mean of the two groups was considered, correctly from the statistical test point of view, as significant. However, statistically significant does not mean automatically biologically significant because a second-order or a side effect could easily be evidenced this way. To help interpretation, the simple graphical representations used here, very close to raw data, were connected through hyperlinks to the tabulated test results. By this means, readers can quickly form their own opinions by viewing the original data.

Additional data files

Tables of the intragenomic P_3 distributions of all species examined, and the PR2-biases between leading and lagging groups for coding sequences, and small and large intergenic regions are available as additional data files in text format with the online version of this paper.

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