# Solving ambiguities in contig assembly of *Idiomarina loihiensis* L2TR chromosome by *in silico* analyses

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## Abstract

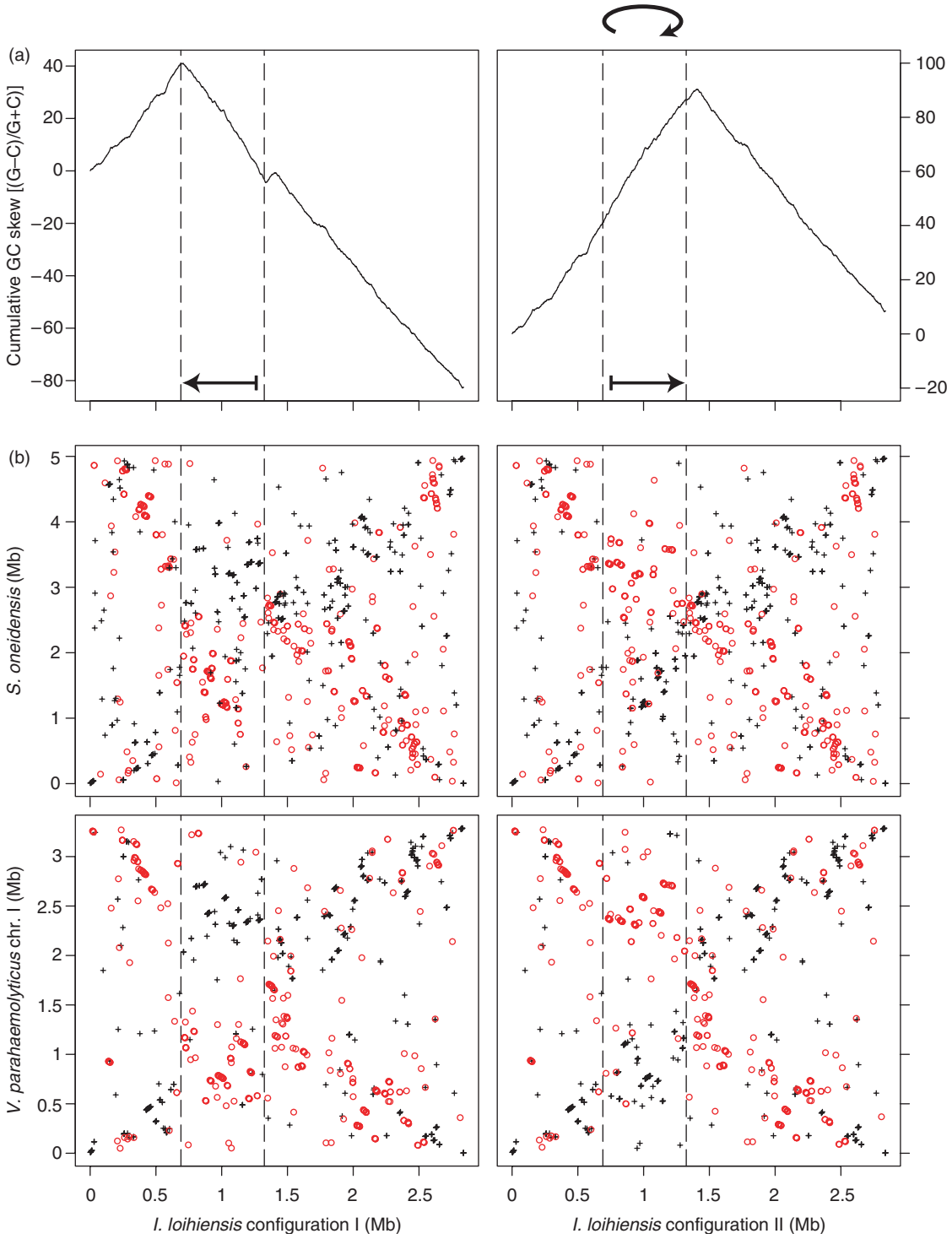
Nucleotide composition analyses of bacterial genomes such as cumulative GC skew highlight the atypical, strongly asymmetric architecture of the recently published chromosome of *Idiomarina loihiensis* L2TR, suggesting that an inversion of a 600-kb chromosomal segment occurred. The presence of 3.4-kb inverted repeated sequences at the borders of the putative rearrangement supports this hypothesis. Reverting *in silico* this segment restores (1) a symmetric chromosome architecture; (2) the co-orientation of transcription of all rRNA operons with DNA replication; and (3) a better conservation of gene order between this chromosome and other γ-proteobacterial ones. Finally, long-range PCRs encompassing the ends of the 600-kb segment reveal the existence of the reverted configuration but not of the published one. This demonstrates how cumulative nucleotide-skew analyses can validate genome assemblies.

## Introduction

In bacteria, bidirectional DNA replication divides circular chromosomes into two arms or replichores (Blattner *et al.*, 1997), delimited by the origin and terminus of replication. Thus, each strand of the double-stranded chromosome is the leading strand of replication on one arm, and the lagging one on the other. The process of replication generates asymmetric nucleotide biases (i.e. different for each DNA strand): directly, by favoring some mutations on one strand, and/or indirectly, since the leading strand encodes most genes (see Frank & Lobry, 1999; Rocha, 2004 for review). More precisely, the leading strand is generally enriched in guanines and depleted in cytosines and, consequently, the lagging strand is enriched in cytosines and depleted in guanines. These biases may be determined by a GC skew, which measures the ratio between the number of guanines and the number of cytosines $[(G-C)/(G+C)]$ on one strand. Due to its guanine enrichment, the leading strand thus presents a positive GC skew and, depleted in guanines, the lagging strand presents a negative GC skew (Lobry, 1996). Therefore, a graphic representation of cumulative GC skews along the chromosome unambiguously reveals localization of the origin and terminus of replication in a large majority of bacterial chromosomes. On this plot, the guanine-rich leading strand displays a positive slope and the guanine-poor lagging strand a negative slope; thus, the origin and terminus of replication correspond to the minimum and maximum of the curve, respectively (Grigoriev, 1998; Frank & Lobry, 2000; Guy & Roten, 2004). Because the length of both replichores is generally similar, and because the published chromosomal sequences generally start at the origin of replication, cumulative GC skew plots of most bacteria present a smooth inverted V-shape (see Fig. 1a, right) (Grigoriev, 1998; Roten *et al.*, 2002).

Homologous recombination occurring between inverted sequences – such as rRNA genes or insertion sequences (IS) – is responsible for most chromosomal inversions. These events can be divided into three categories: (1) symmetrical or (2) asymmetrical inter-arm inversions, and (3) intra-arm inversions. Inter-arm rearrangements are symmetrical (1) when the homologous sequences involved in the recombination are at a similar distance from the origin of replication on each arm of the chromosome. Such rearrangements conserve the inverted V-shape on cumulative GC skew plots, since the length of the arms, the leading or lagging status of the strands and gene orientation relative to replication are conserved. Asymmetrical inter-arm recombination (2) is

similar to (1) (it does not affect the leading or lagging status of the strands nor the gene orientation relative to replication), except that the two inverted recombination sites on each arm of the chromosome are at a different distance from the origin, thus changing the relative lengths of the arms once the rearrangement occurred. As a result, the displaced terminus is not at the middle of the cumulative GC skew plot, which presents an asymmetrical inverted V-shape, i.e. one branch of the inverted 'V' is longer than the other. Finally, some chromosomal inversions occur within a single



arm of the chromosome (3), exchanging a guanine-rich leading strand for a guanine-poor lagging strand. The resulting slope of the inversion is opposite, locally disrupting the symmetrical inverted V-shape, and thus enabling a straightforward detection of such events on cumulative nucleotide skew curves.

Systematic GC skew analyses of more than 400 published bacterial chromosome sequences reveal that asymmetrical inter-arm and intra-arm rearrangements are rare [see Comparative Genometrics website, http://www.unil.ch/comparativegenometrics/ (Roten *et al.*, 2002)]. For instance, *Pseudomonas aeruginosa* PAO1 and *Xylella fastidiosa* 9a5c chromosomes display significantly asymmetrical arm lengths, revealing an inter-arm inversion (2) (Stover *et al.*, 2000; Van Sluys *et al.*, 2003). In the case of *P. aeruginosa*, it appears that the sequenced isolate was not representative of the major bacterial population, which displays classical symmetrical chromosome architecture (Barekzi *et al.*, 2000). In the case of *X. fastidiosa*, a complex rearrangement involving prophages could be the source of the asymmetry in the 9a5c strain (Canchaya *et al.*, 2004). This asymmetry is absent in the Temecula1 strain. Similarly, intra-arm inversions were mostly reported in the highly unstable chromosome of *Yersinia pestis* (Parkhill *et al.*, 2001; Deng *et al.*, 2002), but these events seem to be associated with the genome-reduction phase presently occurring in *Y. pestis* and due to the restriction of its host range (Wren, 2003). In summary, it clearly appears that, when inversions occur, they are most often centered on the origin of replication. This suggests that chromosome configurations presenting replichores of similar lengths and a conservation of gene orientation with respect to replication are favored (Eisen *et al.*, 2000; Tillier & Collins, 2000; Kothapalli *et al.*, 2005). Consequently, cumulative skew curves different from symmetric inverted V-shapes reveal atypical chromosome configurations.

The Comparative Genometrics database (Roten *et al.*, 2002), dedicated to the analysis of whole prokaryotic genomes, includes the nucleotide skew curves for all bacterial chromosomes available at the NCBI database (Wheeler *et al.*, 2004). It enables the detection of chromosomes displaying atypical nucleotide skew patterns, such as the one of *Idiomarina lohiensis* L2TR, isolated from hydrothermal vents in Hawaii. This deep-sea *Gammaproteobacterium*, able to grow in a wide range of temperatures and salinities, seems to draw its energy mostly from amino-acids fermentation, rather than from usual sugar degradation pathways. In this contribution, this atypical chromosome architecture was precisely characterized.

## Materials and methods

### Sequences and document availability

*Shewanella oneidensis* MR-1 and the chromosome 1 of *Vibrio parahaemolyticus* RIMD 2210633 sequences are available on the NCBI website (http://www.ncbi.nlm.nih.gov/) under accession numbers NC_004347 and NC_004603, respectively. *Idiomarina loihiensis* L2TR fasta sequences (first published under accession number NC_006512) and nucleotide counts per 1-kb window for both configurations are available at http://www.unil.ch/comparativegenometrics/idiomarina/index.htm.

### Nucleotide skews

The sequence was divided in 1-kb windows and the GC skew $[(G-C)/(G+C)]$ was calculated for each window. The cumulative GC skew corresponding to the window $i$ is the cumulation of the GC skews from the beginning of the sequence to the window $i$ (Grigoriev, 1998).

### Identification of repeats

The chromosome sequence of *I. loihiensis* was searched for direct and inverted repeats longer than 200 bp with REPuter (Kurtz & Schleiermacher, 1999).

### Gene-order conservation analysis by X-plot

The chromosome sequence of *I. loihiensis* L2TR was compared with the chromosome of two related bacteria: the chromosome of *S. oneidensis* MR-1 and the chromosome 1 of *V. parahaemolyticus* RIMD 2210633. Sequences homologous in genome pairs were identified with PROMER, a program of the MUMMER 3.18 package (Kurtz *et al.*, 2004): a maximal 50-nt gap allowed between two adjacent matches

**Fig. 1.** Nucleotide skews and plots of relative positions of homologous sequences (colinearity) in *Idiomarina loihiensis* and related species. In both panels, the originally published sequence (configuration I) of *I. loihiensis* chromosome is on the left column, and the proposed symmetrical configuration II is on the right column. Vertical bars indicate putative rearrangements sites, which coincide with inverted repeated sequences. Location on chromosomes is given in Mb (*x*-axis on both panels, and *y*-axis in b). (a) Cumulative GC skew of both configurations. Scale indicates the cumulative excess of guanine over cytosine: $[(G-C)/(G+C)]$, calculated in 1-kb windows. (b) Gene-order conservation analysis (X-plot) by comparison of relative positions of homologous sequences in *I. loihiensis* and closely related bacteria *Shewanella oneidensis* and *Vibrio parahaemolyticus* chr. 1. The pairs of homologous sequences are represented on the plot at the intersection of their position on *I. loihiensis* (*x*-axis) and *S. oneidensis* (*y*-axis, top panel) or *V. parahaemolyticus* (*y*-axis, bottom panel) genomes by a black cross if the segment is on the same strand on both chromosomes, and by a red circle if they are on complementary strands.

was selected, combined with a maximal 100-nt extension and a maximal distance of extension attempts of 100 nt. A direct hit is a homologous segment that is located on the same strand (+/+ or −/−) on both compared bacteria, an indirect hit is when paired sequences are located on complementary strands (+/− or −/+). Quality measurement of the X-plots is obtained by correlating the distances of each hit (whether direct or indirect) from the origin of replication on both genomes and by calculating Pearson's correlation coefficient. Comparing a genome with a related strain that has undergone only inversions centered on the origin of replication provides a Pearson's correlation coefficient close to one. Comparing two genomes whose gene orders are completely different provides a coefficient close to zero.

### Long-range PCRs

The Expand Long Template PCR System (Roche) was used to amplify large chromosomal segments. Eight primer sets were used for detecting each configuration (Supplementary Tables S1 and S2). Amplifications were performed according to manufacturer recommendations, with 500 ng of genomic DNA and at a 58 °C annealing temperature. A single common mix containing water, DNA template, PCR buffer, nucleotides and enzyme was used for the 18 PCR reactions. Primers amplifying a 11-kb region encoding general metabolism genes and located outside the 0.6-Mb chromosomal inversion were used as a positive control and water instead of primers as a negative control.

## Results and discussion

The cumulative GC skew curve performed on the 2.8-Mb-long chromosome of *I. loihiensis* L2TR (Hou *et al.*, 2004) displays a nonsymmetrical pattern (Fig. 1a, left), whereas those of almost all other *Gammaproteobacteria* are classical (Supplementary Fig. S1) as discussed above. In the case of *I. loihiensis* L2TR, the curve displays three local extrema: in addition to the usual overall cumulative GC skew maximum at 0.7 Mb, there is a local minimum at 1.3 Mb and a local maximum at 1.4 Mb (Fig. 1a, left). Following the reasonable hypothesis that the terminus of replication is actually located approximately at the middle of the curve, on the local maximum at 1.4 Mb, then the 600-kb chromosome segment located between the two inflexion points of the curve located at 0.7 and 1.3 Mb displays an opposite slope orientation than expected, suggesting a large DNA inversion. Confirming this view, inverted repeated (IR) sequences of 3.4 kb were identified at the boundaries of the putative inversion. These IRs are located between positions 688 321 and 691 693 on one end, and between positions 1 335 927 and 1 332 555 on the other. They display 3349 identical bases out of 3373 and contain genes encoding proteins involved in general metabolism (IL631 to IL633 and IL1241 to IL1243) and an IS2 transposase (IL633.1 and IL1239.1). They are the longest IR sequences in the *I. loihiensis* chromosome, rRNA operons excepted. Because they could be recognized by the homologous recombination machinery, these regions might be directly involved in an intra-arm inversion, explaining the atypical skew pattern of the published *I. loihiensis* sequence. On the other hand, unusually long homologous sequences may also be the source of contig misassemblies. To sum up, since asymmetrical rearrangements are not favored, the existence of a symmetrical chromosome configuration (configuration II), in which the 600-kb DNA segment bordered by the IR is inverted, is proposed.

An *in silico* inversion of this region was consequently performed and both *I. loihiensis* chromosome configurations were compared to other *Gammaproteobacteria* counterparts. Several elements support that the symmetrical configuration II of this study is more likely to be present in natural conditions. First of all, the cumulative GC skew curve resulting from the proposed configuration II now displays a symmetrical inverted V-shape (Fig. 1a, right). Using BLAST analysis (word as 7 and standard gap penalties) (Altschul *et al.*, 1990), we also detected starting at position 1 387 610 (i.e. around the middle of the sequence), a 28-nt sequence (ATTGCGTATAATGTATATTATGTTAAAT) that has 25 nucleotides in common with the *Escherichia coli* K-12 *dif* sequence. In the latter bacteria, the *dif* sequence is involved in the resolution of chromosome dimers, which occurs in the terminus region and is apparently well conserved throughout the bacterial phylogenetic tree (for review see Lesterlin *et al.*, 2004). The presence of this sequence supports the proposition made above that, as in the large majority of bacterial chromosomes, the terminus of replication is localized at the maximum of the cumulative GC skew curve in the symmetrical configuration II. Moreover, the terminus of replication is actually at the middle of the chromosome sequence, separating it into two replichores of similar sizes. Another supporting piece of evidence, given that the terminus was correctly identified at 1.4 Mb, is that one out of four rRNA operons would be antioriented – i.e. transcribed in the opposite direction than the replication – in the published configuration, an extremely rare situation (Guy & Roten, 2004). Furthermore, the gene order of both *I. loihiensis* configurations was compared with two related bacteria, *S. oneidensis* and *V. parahaemolyticus* chr. 1 (Fig. 1b). The comparison of the symmetrical configuration II (Fig. 1b, right) with related bacteria is closer to the typical X shape (see Tillier & Collins, 2000, for examples), than the comparisons of configuration I with the same related bacteria (Fig. 1b, left). Consequently, Pearson's correlation coefficients of the X-plots are significantly higher in configuration II (Supplementary Table S3).

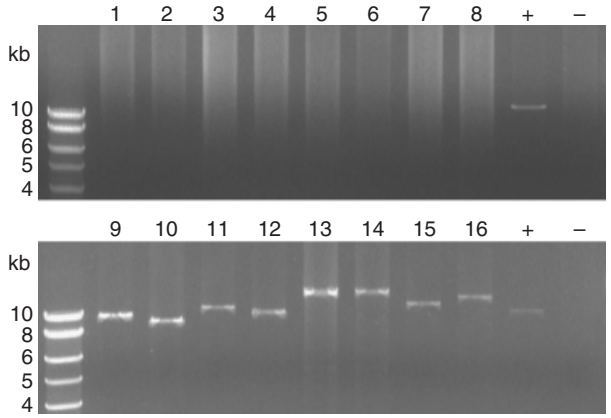



**Fig. 2.** Long-range PCR amplifications of *Idiomarina loihiensis* chromosome in configurations I and II. Eight primer sets were used for detecting each conformation (Supplementary Tables S1 and S2). Primers amplifying a 11-kb region located outside the 0.6-Mb chromosomal inversion were used as a positive control and water as a negative control, respectively. Lanes 1–8 and 9–16, detection of published configuration I and symmetrical configuration II, respectively. Lanes + and −, positive and negative control, respectively.

Finally, to validate experimentally the existence of the symmetrical chromosome configuration II in the *I. loihiensis* genome, primers encompassing each copy of the 3.4-kb IR were designed. Owing to the large size of repeated sequences, long-range PCR reactions were performed using combinations of primers able to promote the amplification of either the published or the symmetrical chromosome configurations (configuration I or II, respectively) on extracted chromosomal DNA from cultures of the *I. loihiensis* DSMZ reference strain (DSM 15497). PCR reactions clearly demonstrate that the symmetrical configuration II is present in the DNA sample (Fig. 2). On the contrary, although each configuration was tested using eight primer pairs, no amplification specific to the published configuration I was detected in manufacturer conditions, including when a DNA sample provided by Hou and coworkers was used (data not shown). Nevertheless, the presence of configuration I in the DNA sample cannot be completely excluded, but, if present, its proportion would be too low to be detected via long-range PCR. The results of the PCR amplifications strongly suggest that the published configuration I was not representative of the *I. lohiensis* population.

In summary, it is concluded that the published sequence of *I. loihiensis* L2TR represents, at best, a rare chromosomal configuration, and that typical *I. loihiensis* isolates display the symmetrical configuration II of this study. An alternative explanation is that the unusual skew pattern of *I. loihiensis* results from the misassembly of some contigs: indeed, for rearrangements implying long inverted repeated sequences, standard PCR verification of contig alignment (10 kb-long PCRs, with a 1 kb overlap) could be unable to identify misassemblies or assembly of minor configurations. Cumulative GC skew plots were already able to detect such assembly problems in *Bifidobacterium longum* NCC2705 (Guy *et al.*, 2005). Standard GC skews are widely used in genome projects. However, the cumulative representation of GC skew seems thus to be more readable and more easily interpreted by molecular biologists not trained in bioinformatics and represent a highly useful complement for detecting architecture anomalies in bacterial chromosomes. Moreover, this fast and simple method may be performed on raw sequence files: neither sequence annotation nor comparison with closely related species is required. Since a large majority of bacteria and more specifically almost all *Gammaproteobacteria* display typical cumulative GC skew curves, the information provided by this genometric tool should be intensively exploited in bacterial genome-sequencing projects, especially for those implying taxa in which no or few other complete sequences are available. Thus, it is proposed that these more informative cumulative skews should be used routinely in addition to the usual noncumulative circular representation (e.g. Andersson *et al.*, 1998) in bacterial-sequencing projects.

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## Supplementary material

The following supplementary material is available for this article:

**Table S1.** Primers used for long-range PCR.

**Table S2.** Primer combinations used to obtain an amplification of either configuration.

**Table S3.** Pearson's coefficients for the correlation of gene orders between the compared genomes.

**Fig. S1.** Cumulative GC skews of two *Gammaproteobacteria*, related to *I. loihiensis* L2TR.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1574-6968.2007.00714.x (This link will take you to the article abstract).

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