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Genome Engineering in *Vibrio cholerae*: A Feasible Approach to Address Biological Issues

Marie-Eve Val1,2, Ole Skovgaard3, Magaly Ducos-Galand1,2, Michael J. Bland1,2, Didier Mazel1,2*

1 Institut Pasteur, Unité Plastïcité du Génome Bactérien, Département Génomes et Génométhique, Paris, France, 2 CNRS, URA2171, Paris, France, 3 Department of Science, Systems, and Models, Roskilde University, Roskilde, Denmark

Abstract

Although bacteria with multipartite genomes are prevalent, our knowledge of the mechanisms maintaining their genome is very limited, and much remains to be learned about the structural and functional interrelationships of multiple chromosomes. Owing to its bi-chromosomal genome architecture and its importance in public health, *Vibrio cholerae*, the causative agent of cholera, has become a preferred model to study bacteria with multipartite genomes. However, most in vivo studies in *V. cholerae* have been hampered by its genome architecture, as it is difficult to give phenotypes to a specific chromosome. This difficulty was surmounted using a unique and powerful strategy based on massive rearrangement of prokaryotic genomes. We developed a site-specific recombination-based engineering tool, which allows targeted, oriented, and reciprocal DNA exchanges. Using this genetic tool, we obtained a panel of *V. cholerae* mutants with various genome configurations: one with a single chromosome, one with two chromosomes of equal size, and one with both chromosomes controlled by identical origins. We used these synthetic strains to address several biological questions—the specific case of the essentiality of Dam methylation in *V. cholerae* and the general question concerning bacteria carrying circular chromosomes—by looking at the effect of chromosome size on topological issues. In this article, we show that Dam, RctB, and ParA2/ParB2 are strictly essential for chrl origin maintenance, and we formally demonstrate that the formation of chromosome dimers increases exponentially with chromosome size.

Introduction

Bacteria were long thought to have a simple genome architecture based on a unique circular chromosome, and it is only in the late 1980s that the first prokaryote with multiple chromosomes, *Rhodobacter sphaeroides*, was characterized [1]. Since this seminal observation, many other species possessing multiple circular or linear chromosomes have been characterized across numerous bacterial lineages [2]. More than 80 multipartite bacterial genomes have been sequenced, propagating various hypotheses to explain their extant nature and posing fundamental questions about the selective benefit of such a genome architecture.

Numerous studies have established the cholera pathogen, *Vibrio cholerae*, as the model for bacteria with multipartite genomes [3]. The genome of *V. cholerae* N16961 consists of two circular chromosomes, a primary 2.96 Mbp chromosome (chrI) and a secondary 1.07 Mbp chromosome (chrII). *V. cholerae*’s genes are asymmetrically distributed between the two chromosomes [4]. ChrI has low interspecies sequence variability and harbors many genes coding for essential biosynthetic pathways. ChrII contains many more species-specific genes, unknown ORFs and proportionally fewer essential genes [4–5]. Furthermore, *V. cholerae*’s particular genomic organization and genetic disparity is consistent within the *Vibrionaceae* family [6–8]. The unusual genome structure of *V. cholerae* has inspired numerous studies to better understand the mechanisms and purposes of maintaining such a genomic organization, resulting in an impressive body of experimental data [9–20]. To date, however, and despite the impressive collective effort of the cited studies along with other research on chromosome and plasmid maintenance systems, the mechanisms coordinating the maintenance of multiple chromosomes are largely unknown. In tackling such pervasive yet fundamental questions, we decided to construct a unique genetic tool allowing targeted massive chromosomal rearrangements in proteobacteria. We applied this powerful technique to answer two outstanding questions. Firstly, we addressed the specific case of the essentiality of Dam methylation in *V. cholerae*. Secondly, we focused our genetic system on more general questions concerning bacteria with circular chromosomes by examining the effect of chromosome size and genetic distribution on topological issues.

Unlike eukaryotic organisms, where chromosomes are managed by common machineries which coordinate up to 90 chromosomes [21], *V. cholerae* has evolved a relatively complex and highly targeted strategy involving interplay of specific and common machineries for the maintenance of each chromosome. Replication of each *V. cholerae* chromosome is controlled by a unique initiator molecule [11]. ChrI replication is initiated at oriI by
DNA methylation by the Dam methyltransferase is essential for chromosome maintenance in various bacterial species, including *Vibrio cholerae*. Dam methylates the palindromic GATC sites in DNA, which become protected from nucleases and thus prevent the degradation of chromosomes during cell division. In *V. cholerae*, Dam methylation is not essential for chromosome segregation, but it is required for the viability of the organism, particularly under conditions of replication stress or nutrient limitation. Dam methylation is also involved in the regulation of gene expression by controlling access to regulatory sites in the genome.

**Author Summary**

*Vibrio cholerae*, the causative agent of cholera in humans, has two circular chromosomes of uneven size, each with distinct maintenance requirements. This is in contrast to classical, *Escherichia coli*-centric bacterial models of a single chromosome. In this study, we took advantage of *V. cholerae*’s atypical genome structure to address important biological issues related to the maintenance of multipartite genomes. We further used *V. cholerae* to determine how genome architecture and genetic organization affects the odds of topological difficulties arising during replication. Our approach consisted of performing massive genome rearrangements to create various synthetic mutants of *V. cholerae* with nearly identical genetic backgrounds. We created mutants of *V. cholerae* with a single chromosome, with two chromosomes of equal size, or with identical origins of replication. To do so, we developed a genetic engineering tool based on the multiplexing of two site-specific recombination systems to allow efficient and directional manipulations of any DNA segment. In this study, we show that Dam, RctB, and ParA2/ParB are only essential for chrI origin maintenance, and we demonstrate that the odds of forming chromosome dimers exponentially increases with chromosome size.
specific genome rearrangements to directly study biological systems in their endogenous host. We developed a genetic tool based on two distinct site-specific recombination machineries, which allow targeted, oriented and reciprocal DNA exchanges throughout the genome. We used *V. cholerae* as a bi-chromosomal bacterial model to show the power of our genetic tool and how its use can help address important biological questions. Using this strategy, we examined the requirement of *Vibrio*-specific essential factors involved in chromosome maintenance for which functions could not be strictly attributed to a specific chromosome. We also investigated the correlation between chromosome size and the rate of formation of chromosome dimers that are the inevitable by-products of frequent recombination associated with recombinational DNA repair. To address all these questions, we created a mutant of *V. cholerae* with all its genetic content reorganized onto a single chromosome. We further refined our study by making additional chromosomal rearrangements to individually decipher each biological issue. In this article, we show that Dam, RctB and ParA2/ParB2 are only essential for chrII origin maintenance. We further demonstrate that the odds of forming chromosome dimers exponentially increases with chromosome size.

**Results/Discussion**

One from two: Reorganizing the genome of *V. cholerae*

We generated a mutant of *V. cholerae* with all its genetic content reorganized onto a single chromosome. To do so, we fused chrI with chrII in a calculated and conservative manner respecting known criteria for chromosome organization and maintenance. Prokaryotic genomes show intolerance towards various chromosome rearrangements such as inversions or relocations of DNA fragments [34–44]. Nevertheless, bacterial chromosomal structure can be drastically altered [45–48] provided that organizational features are respected (for reviews [49–51]). The fused chromosome was constructed to conserve the “ori-ter” axial symmetry, gene synteny, strand bias and the polarities of the original replichores. Replication of the fused chromosome initiates at ori of chrI and finishes in the terminus of chrII near dfy2. The single fused chromosome carries exclusively chromosomal-like attributes for replication and chromosome segregation (ori, ParA1/B1, dfy2), like other mono-chromosomal bacteria. By initiating replication at oriI, we conserve the replication-associated gene dosage on chrI [10]. Lastly, comparative genomics has shown that the ter region of chrI is flexible and would likely tolerate the integration of the 1 Mb chrII [7,52].

To perform the above-mentioned genome rearrangements, we developed a genetic tool which allows efficient and directional manipulations of any DNA segment. It involves two site-specific recombination systems which normally promote precise excision of the temperate phage genomes, λ and HK022, from their chromosomal location [53]. We used λ and HK022 integrases (IntIλ and IntIHK), their respective excision factors (Xisλ and XisHK) and their associated left and right excision sites (attRλ/attLλ and attRHK/attLHK). Unlike other site-specific recombination systems used for precise genome manipulation such as Crec/loxP [54] or Flp/FRT [55], the λ and HK022 recombination reactions have the calculated advantage of being directionally controlled, as the presence of the Xis excision factors orientates the catalytic reactions in one direction. This characteristic is very useful for two reasons: first, it insures that the mutant strain will not revert to the wild-type configuration after chromosomal rearrangement. Second, the newly formed sites (attB/P) react poorly with the substrate sites (attR/L) [56]. Therefore the same system can be reused in the mutant strain to perform new rearrangements at other positions by integrating new attR/L sites. In theory, this system could be used an infinite number of times in the same strain.

To fuse the two chromosomes of *V. cholerae*, each partner attL and attR sites specific to the same integrase were inserted on separate chromosomes: attRHK/attLλ were inserted at the junction between the two replichores in the terminus region of chrI and attLHK/attRL were placed flanking [parA2/B2-oriII-rctA/B] in the origin region of chrII (Figure 1A). The consecutive recombination reactions between attRHK/attLHK and attRL/attLλ sites, upon expression of Int and Xis, led to the fusion of chrI with chrII (Figure 1B, 1C). To visualize chromosomal rearrangement events, we used a colorimetric screen based on recombination-dependent reconstitution and expression of the lacZ gene (Figure 1E). We obtained a stable MonoCHromosomal *V. cholerae* mutant strain (MCH1) with a single chromosome of the expected 4 Mbp size (Figure 1F) observable by pulsed field gel electrophoresis (PFGE). MCH1 cells attain a generation time of 29 minutes when grown in fast-growing conditions (Table S1). Under the microscope, MCH1 fixed cells are indistinguishable from N16961 wild-type (WT) (Figure 1G) and the counting of viable cells forming microcolonies confirmed that MCH1 incurs no increase in the rate of mortality compared to the WT (data not shown). We measured the DNA distribution in exponentially growing cultures by flow cytometry and compared these distributions with modeled distributions (Figure S1). Whereas WT has a replication pattern which can be successfully modeled by assuming that chrII initiates late and terminates at approximately the same time as chrI as previously described [16], our analysis of MCH1’s replication pattern was consistent with a single chromosome replicated at a constant rate (Figure S1).

**RctB initiator and ParA2/B2 partitioning factors are essential for chrII maintenance only**

We have taken a radical genetic approach by rearranging the genome of *V. cholerae* to investigate the specific biological functions of RctB and ParA2/B2. Since chrII is indispensable, these factors, essential for chrII initiation and partition, are ultimately essential for cell viability [11,20,22]. However, an additional role in the maintenance of chrI could never be formally tested due to the essentiality of their functions for chrII perpetuation. Recombinational fusion of the two chromosomes in MCH1 resulted in the excision of an 8 kb circular molecule carrying [parA2/B2-oriII-rctA/B-aph] (Figure 1C). The excised molecule encoded a functional aph gene conferring kanamycin resistance to the parental strain of MCH1, MV127. This circular molecule was readily lost in absence of selection observable by the absence of kanamycin resistance in MCH1 cells (Figure 1D). Loss of the 8 kb molecule was further confirmed by PCR, showing an absence of amplification of parB2 and rctB loci from MCH1 genomic DNA, while these loci could normally be amplified from MV127 genomic DNA (data not shown). Loss of the 8 kb molecule was surprising since it harbored the oriII origin of replication and a centromere-like parS2-B site (within rctA) [57] along with associated replication (rctA/B) and partitioning (parA2/B2) factors that should allow it to replicate autonomously in the cell. We have no experimental evidence that could explain this loss, but it could be the result of partition-mediated incompatibility [58] between parS2 sites located on separate entities, the fused chromosome and the 8 kb circular molecule. Yet, by physically linking chrII to chrI in MCH1, we placed replication and partitioning of chrII under the control of chrI machinery rendering chrII factors for replication initiation (RctB) and partitioning (ParA2/B2) non-essential.
Massive Genome Rearrangements in *V. cholerae*

Most of the centromere-like *parS2* sites are located near *oriII*, ensuring its partition, but a functional *parS2* site, *parS2-1*, was found located near the chrI terminus [57]. Therefore, ParA2/B2 could have an important function for the segregation of the terminus region of chrI. Under the microscope, MCH1 cells are indistinguishable from WT (Figure 1G). Nucleoid staining with DAPI shows no evident segregation or division problems that would be easily detectable by the presence of anucleoid cells, filaments and chromosomes trapped in the septum of division (Figure 1G). Our approach allowed us to readily demonstrate that the essential functions of RctB and ParA2/B2 in *V. cholerae* are strictly limited to chrII maintenance.

The essential activity of Dam is restricted to replication initiation at *oriII*

All previous in *vivo* Dam studies were undertaken in *E. coli*, where Dam is not essential. Here we investigate the essential function of Dam directly in *V. cholerae* to eliminate confusion arising from extrapolated results from *E. coli*. MCH1 enabled us to test the essentiality of Dam in replication initiation, since it only carries a single origin of replication, *oriI*. We deleted *dam* in MCH1 and the WT. Deletion of *dam* was done in the presence of pGD93, a complementing temperature sensitive replicating plasmid expressing *V. cholerae dam* under the control of an arabinose-inducible (permissive conditions) and glucose-repressible (restrictive conditions) promoter [9]. In the presence of Dam, both WTΔ*dam*-pgGD93 and MCH1Δ*dam*-pgGD93 grew normally (Figure 2A). Under restrictive conditions when Dam was depleted, WTΔ*dam* colonies were hardly visible (Figure 2B) confirming the essentiality of Dam in *V. cholerae*. MCH1Δ*dam*, on the other hand, grew and formed colonies under restrictive conditions (Figure 2B), indicating that Dam is no longer essential. This result demonstrates that initiation of replication at *oriI* doesn’t require Dam. To more precisely characterize the role of Dam, we created a second mutant of *V. cholerae* where we maintained two distinct chromo-

![Figure 1. A mono-chromosomal *V. cholerae* model, MCH1. A. *V. cholerae* MV127 strain with att*R* sites from L and HK022 phages inserted at precise loci. Recombination sites [att*R* and att*L] replaced *dfl* on chrI and [att*L and att*R*] flanked *parAB2-oriII-rctAB* on chrII. B. Recombination [att*R* × att*L] and [att*L × att*R*] mediated by the expression of IntX*6* and IntX*6HK*6. C. Recombination events [att*R* × att*L] regenerate lacZ, allowing for phenotypic detection of rearranged chromosomes. D. Without selection, the 8 kb excised molecule (carrying a kanamycin resistance gene) was lost. E. Blue sector appearing within single conjugant on X-Gal supplemented LB-agar plates indicates recombination events between [att*R* × att*L]. F. Ethidium bromide stained PFGE of genomic DNA: Lane 1, *S. pombe* marker (BioRad); Lane 2, WT (N16961); Lane 3, MCH1. G. Microscopic observation of WT (top panel) versus MCH1 (bottom panel). Nucleoids of exponentially growing cells stained with DAPI (green) merged with phase-contrast images (red). doi:10.1371/journal.pgen.1002472.g001](https://www.plosgenetics.org/doi/10.1371/journal.pgen.1002472.g001)
Figure 2. Dam is only essential for replication initiation of chrII from oriII. Growth of WT Δdam/pGD93, MCH1 Δdam/pGD93 and ICO1 Δdam/pGD93 on LB-agar plates under (A) permissive conditions (+0.2% arabinose at 30°C, allowing dam expression and pGD93 replication) or (B) restrictive conditions (+1% glucose at 42°C, repressing dam and preventing pGD93 replication). doi:10.1371/journal.pgen.1002472.g002

viable, this precisely defined oriII as the region where Dam executes its essential function. This result substantiates earlier in vitro work showing that RctB preferentially binds methylated oriII [9]. We propose that in absence of Dam, GATC sites in oriII do not become methylated, preventing the binding of RctB to oriII and therefore precluding chrII replication initiation and maintenance which is fatal to the cell.

Chromosome dimer formation increases exponentially with the size of the chromosome

Formation of dimeric chromosomes is a particular problem associated with the circularity of bacterial chromosomes. We used V. cholerae as a bacterial model to determine how genome architecture affects the odds of topological difficulties during replication by assaying the effect of chromosome size on the rate of chromosome dimer formation. Very few cells carrying a dimer are expected to yield viable progeny in the absence of resolution. Inactivation of chromosome dimer resolution in E. coli results in ~15% cell death per generation, which corresponds to the estimated rate of chromosome dimers formed at each cell generation [60–61]. We measured the fitness defect of a dif mutant by growth competition experiments, in which the growth of the mutant strain was directly compared to the growth of its parent (Figure 3B) to quantify the rate of dimers formed on a dif-carrying chromosome. In V. cholerae WT, 8.8% of dimers per cell per generation are formed on the 3 Mbp chrI (Δdif1) and 3.4% of dimers are formed on the 1 Mbp chrII (Δdif2) when grown in rich LB media (Figure 3A, 3B). In MCH1, 12.5% of dimers per cell per generation are formed on the 4 Mbp chromosome (Δdif2) under the same growth conditions (Figure 3A, 3B). These results suggested that dimer formation increases with replicon size. To strengthen our interpretation, we decided to construct an additional mutant of V. cholerae with two equally sized chromosomes of 2 Mbp and measure the rate of dimer formation on each chromosome. We transferred 1 Mbp from chrI to chrII by swapping the 1.05 Mbp DNA fragment evenly surrounding dif1 with the 0.12 Mbp DNA fragment evenly surrounding dif2, resulting in the exchange of dif1 and dif2 using the genetic tool described above (Text S1, Figure S2). We obtained a mutant of V. cholerae with Equally Sized Chromosomes (ESC1 with chrI/II and chrII/I) observable by PFGE (Figure S2D). A measure of the rate of chromosome dimers formed on the two 2 Mbp chromosomes was performed in ESC1. Our results show that 4.9% of dimers per cell per generation are formed on the 2 Mbp chrI/II (Δdif2) and 4.3% of dimers are formed on the 2 Mbp chrII/I (Δdif1) (Figure 3A, 3B). We plotted the rate of chromosome dimer formation as a function of chromosome size and observed a linear relationship between chromosome size and the logarithm of the frequency of dimer formation (r² = 0.97) (Figure 3C, Methods). This result indicates that chromosome dimer formation increases exponentially with the size of the chromosome. In ESC1, the two equally sized chromosomes, chrI/II and chrII/I, have an asymmetric distribution of genes, specific machineries for their respective maintenance, distinct terminus regions and, very certainly, distinct chromosome structure, and yet the probability of dimer formation for each chromosome is essentially equivalent (Figure 3A). This implicated chromosome size as the primary influence on the rate of dimeric chromosome formation in an identical genetic background.

Homologous recombination involves a Holliday junction intermediate which is resolved by the RuvABC complex leading to either crossover or non-crossover potential products with only crossovers leading to the formation of chromosome dimers [62]. In E. coli, the RuvABC pathway is biased towards generating non-crossover products [63–64]. Since this bias can vary between species, it is not possible to infer the effects of genome architecture on the formation of chromosome dimers by direct comparison between bacteria with single and multiple chromosomes or between bacteria with multiple chromosomes of different sizes. V. cholerae allowed us to modify the size of the chromosomes by transferring DNA from one chromosome to the other, with minimal modifications of the genetic background.

Genetic information distribution between the two chromosomes impacts chromosome dimer formation

We tested the effect of DNA distribution between multiple chromosomes on the total rate of chromosome dimer formation. To do so, we measured the fitness defect of xerC mutants to obtain a quantification of the total rate of chromosome dimers formed in the cells (Figure 3D). As a consequence of more dimer formation in WT compared to ESC1, we observed that a xerC deletion had a greater effect on WT than on ESC1 (15.5% in WT, 10.8% in ESC1). The unequal (WT) or equal (ESC1) genetic distribution influences the chances for chromosome dimers to arise. Based on this result, it might be considered surprising that the extant WT genome configuration has been selected and all vibrios characterized to date have been shown to possess two unequally sized chromosomes [8]. This suggests that dimer formation has little impact on the selection of DNA distribution on multiple chromosomes.
Massive Genome Rearrangements in *V. cholerae*

| strain | chromosome | chromosome size (Mbp) | origin | terminus | f dimer (%) |
|--------|------------|-----------------------|--------|----------|-------------|
| WT     | chr I      | 2.96                  | oril   | dif1     | 8.8 (+/- 0.5) |
|        | chr II     | 1.07                  | orill  | dif2     | 3.4 (+/- 0.4) |
| MCH1   | chr I+II   | 4.03                  | oril   | dif2     | 12.5 (+/- 0.6) |
| ESC1   | chr I / II | 2.04                  | oril   | dif2     | 4.9 (+/- 0.4) |
|        | chr II / I | 2.00                  | orill  | dif1     | 4.3 (+/- 0.3) |

**B**

Number of generations

**C**

LOG10 (f dimer/parent) vs. chromosome size (Mbp)

**D**

f total dimer (%)
Massive Genome Rearrangements in *V. cholerae*

Figure 3. Chromosome dimer formation increases exponentially with the size of the chromosome. A. Table summarizing the chromosomal features of WT, MCH1 and ESC1. Frequency of dimers formed on each chromosome per cell per generation (fDimer) in percent (+/− standard error of the mean). B. Growth competition experiment to assay the activity of dif sites in chromosome dimer resolution. The logarithm of the ratio between the number of CFUs obtained with strains carrying dif sites and the number of CFUs obtained with their isogenic *M*diff strains, is plotted as a function of the number of generations. Shown is the plot corresponding to the mean ratio of three independent experiments. C. Logarithm of the frequency of chromosome dimer formation plotted as a function of chromosome size. Dots display means of three independent experiments (+/− standard error of the mean). D. Histograms representing the total rate of chromosome dimers formed in WT and ESC1. Bar display the means of three independent experiments (+/− standard error of the mean) of the frequency of cells that the mutant strains (ΔxerC) failed to produce at each generation compared to their parents (in percent).

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One possible explanation for *V. cholerae*’s unequally sized replicons and distinct replication initiation mechanisms may lie in adjusting the balance between genes found on separate chromosomes in response to drastic changes in growth conditions [10,17,65]. Gene dosage tends to shape chromosome organization of fast-growing bacteria, favoring placement of genes involved in translation and transcription near the origin of replication [65]. Differential gene dosage depends on replication rate, chromosome size and doubling time. This effect is particularly important for *V. cholerae* with its two chromosomes of uneven size and extremely short generation time. Indeed, when *V. cholerae* growth rate increases, origin-proximal loci of chrI are amplified by up to four copies per cell, yet origin-proximal loci of chrII never total more than two copies per cell [17]. Consistent with its larger size, gene dosage effects on chrI are greater than on chrII [10,16]. Differently sized replicons may thus be selectively advantageous as a means to allow for a more nuanced gene dosage effect. This is certainly the case for the vibrios, where a higher abundance of growth-essential and growth-contributing genes are located near the origin of replication of chrI coupled with a dearth of such genes on chrII. This theory lends itself well to further investigation using our genetic engineering tools.

**New insights into bacterial genome organization**

We developed a site-specific recombination-based engineering tool, which provides us with a powerful means to massively reorganize in principle any prokaryotic genome provided that necessary host factors are present. This genetic tool consists in harnessing the λ and HK022 recombination systems to perform a large panel of genome reorganizations. By controlling the location and the orientation of each partner recombination site, we can obtain a large variety of genome rearrangements, such as chromosome fusion (e.g. MCH1), transfer and exchange of DNA fragments (e.g. ESC1), deletion, insertion, inversion or substitution of DNA (e.g. ICO1). Thanks to the construction and analysis of various synthetic mutants, we were able to tackle important biological issues on chromosome maintenance in *V. cholerae*. We showed that Dam, RecB and ParA2/ParB2 factors are essential for chrII maintenance. We further revealed that the odds of forming chromosome dimers exponentially increase with the size of a circular chromosome.

Our construction of mutants with massive genome rearrangements demonstrates the incredible plasticity of prokaryotic genomes. All of these genomic mutants preserved the rapid growth characteristic of vibrios, although with a slightly extended generation time (Table S1) that may be linked to their alternative genomic structure. This is currently under investigation. Recent advancements in the field of synthetic biology have demonstrated that the *de novo* creation of artificial genomes is now an attainable objective [66]. The recent assembly of the 580 kb genome of *Mycoplasma genitalium* starting from chemically synthesized oligonucleotides [67] and the successful demonstration that one can maintain and engineer a bacterial genome in a yeast and then transfer it to a bacterial recipient cell to generate an engineered bacterium [68] pave the way for many applications previously thought to be out of reach [69]. The current understanding of bacterial genomic organization and its connection with precise phenotypic properties is insufficient to propose an optimized genome arrangement to the field of synthetic biology. MCH1 is by far the closest isogenic mono-chromosomal model that can be used to make comparisons with the bi-chromosomal *V. cholerae* N16961 strain. A previous work has been done in *Sinorhizobium meliloti*, in which spontaneous fusions of the three natural replicons occurs at low frequency through recombination between repeated sequences in the genome [70]. In these experiments, the three different fused molecules all conserved their functional origins of replication, and the resulting fusion was reversible, rendering the results inconclusive in terms of the relationship between growth advantage and genome organization. On the contrary, the single chromosome of our engineered MCH1 is stable, contains only a single origin and terminus of replication and therefore provides us with a powerful new tool to investigate the selective advantage(s) of the characteristic multipartite genome organization of vibrios. New insights into bacterial genome organization and determination of how genomes are arranged can help us to design more optimized chromosomes, which will undoubtedly open novel developments in the field of synthetic biology.

**Methods**

**Bacterial strains and growth conditions**

Bacterial strains and plasmids used in this study are listed in Table S2. Cells were grown at 37°C in Luria broth. Antibiotics were used at the following concentrations: ampicillin, 75 μg/mL; chloramphenicol, 25 μg/mL for *E. coli* and 5 μg/mL for *V. cholerae*; kanamycin 25 μg/mL; spectinomycin 100 μg/mL; zeocin 25 μg/mL. Diaminopimelic acid was used at 0.3 mM, X-Gal (40 μg/mL); IPTG (1 mM); arabinose (0.2%) and glucose (1%).

**General cloning procedures**

DNA cassette containing the *att* recombination sites were transferred from a plasmid vector to the chromosome by two homologous recombination steps. To provide homology for integration, two 500 bp regions spanning the point of insertion were amplified from N16961 chromosomal DNA by PCR. The amplified fragments were cloned into an R6K-γ-ori-based suicide vector, pSW7848 that encodes the ccdB toxin gene under the control of an arabinose-inducible and glucose-repressible promoter, P<sub>BAD</sub>. The sequences containing the *att* recombination sites of interest were then cloned between the two chromosomal fragments. For cloning, pTTS13 was used as a plasmid host [71]. For conjugal transfer of plasmids to *V. cholerae* strains, *E. coli* B3914 was used as the donor [71]. Selection of the plasmid-borne drug marker resulted in integration of the entire plasmid in the chromosome by a single crossover. Elimination of the plasmid-borne backbone resulting from a second recombination step was selected for by arabinose induction of the ccdB toxin gene.
MCH1 construction

V. cholerae N16961 El Tor strain deleted for lacZ was used to create the mono-chromosomal MCH1 strain [4,72]. Following the above-mentioned cloning and genome engineering procedures, four attR/L sites were inserted at precise chromosomal loci near dfl on chrI and near orfII on chrII using pSW784@-derivatived KO vectors pMP36 (attR3), pMP42 (attL3), pMP55 (attRHK), pMP49 (attLHK) (Table S2). First, [attR3, 3’ lacZ-FRT-aph-FRT] was inserted downstream of dfl on chrII using pMP36 in N16961 ΔlacZ generating strain MV121. The aph cassette was excised using pCP20 for expression of Flp recombinase that catalyzes recombination between the two FRT sites [73–74]. After Flp-mediated recombination, a single FRT site remained near orfII and the strain become sensitive to kanamycin, MV122Δaph. Second, [attL3, 5’ lacZ-FRT-aph-FRT] was inserted upstream of dfl on chrI using pMP42 in MV122Δaph generating strain MV124. The aph cassette was excised using pCP20, generating the mutant MV124Δaph. We checked MV124Δaph by PCR to make sure that undesirable recombination events between the remaining FRT site on chrII with FRT sites on chrI didn’t occur. Third, dfl was replaced by [attRHK-FRT-aph-FRT] using pMP35, yielding strain MV125. To insert attRHK close to dfl, it was necessary to delete dfl to prevent site-specific integration of a dfl-carrying KO-vector mediated by the endogenous V. cholerae XerC/D recombinases [19]. The aph cassette was not excised, this antibiotic resistance cassette serving as a reporter to follow the subsequent loss of the excised 8 kb circular molecule resulting from the fusion of chrI with chrII. Fourth, [attLHK] with no antibiotic resistance cassette was inserted downstream of parB2 using pMP49 generating mutant MV127 (Figure 1A).

A temperature-sensitive replicating vector pMP6 expressing intL xisL, mtagG-xb isxL was conjugated into MV127. Donor cells B2163 (pMP6) and recipient cells (MV127) were conjugated for one hour at 30°C and plated on LB-agar at 30°C supplemented with ampicillin, X-Gal and IPTG to select for pMP6 and monitor recombination events between attL3 and attR3. Reconstitution and expression of the β-galactosidase encoding gene led to appearance of blue cells when grown in presence of X-Gal and IPTG. After 36 hours of growth at 30°C, blue quarters appeared within single white conjugant colonies (Figure 1E). From blue/white colonies of mixed population, cells were grown at 30°C in LB in presence of ampicillin to enrich for chromosome rearrangements. Cells were plated on LB supplemented with X-Gal, IPTG to monitor attL3 and attR3. Recombination events and incubated at 42°C to cure pMP6. All selected colonies were completely blue. Ten clones were isolated and tested by PCR using primers flanking both recombined attB3 and attB1HK sites to verify that recombination occurred between all four recombination sites. All tested blue clones also had recombined attRHK×attLHK. Fusion of the two chromosomess resulted in the excision of an 8 kb circular molecule. In absence of antibiotic pressure that selected for this 8 kb circular molecule (aph gene formerly located in the terminus region of chrI), the molecule was rapidly lost. All remaining and undesired FRT and attP sites were excised within the 8 kb molecule and subsequently lost. The resulting mutant carries a single circular chromosome, free of antibiotic resistance cassettes and containing only two short 50 bp attB sites that delimit chrI from chrII. Genomic stability of the mutant was established over 1000 generations carried out during a long-term evolution experiment.

Pulsed field gel electrophoresis

The preparation of genomic DNA embedded in agarose gels and the protocol for PFGE was performed as previously described [8,75].

Dam depletion

WT, MCH1 and ICO1 strains were deleted for dam using pGD121 knock-out vector in the presence of pGD93 (Dam complementing vector) and then depleted for Dam as previously described [9].

Growth competition assay

The proportion of cells that a mutant strain deficient in dimer resolution fails to produce at each doubling time of its parent can be measured by growth competition experiments. Growth competitions of V. cholerae strains are described in [19]. V. cholerae cells were grown at 37°C with a 10−3 dilution in LB media every 8–12 h. Colony-forming units (CFUs) of mutant and parental cells in the cultures were determined by plating on appropriate antibiotic plates. These numbers were used to calculate the number of generations of the parent cells between each time point and the CFUs ratio of mutant versus parent cells at each time point. This ratio varies exponentially with the number of generations. The coefficient of this exponential is a good estimation of the rate of dimer formation [19]. Following this method, we estimated the rate of dimer formation for each mutant in three independent experiments. In Figure 3C, the relationship between the rate of dimer formation and the logarithm of chromosome size has a very high R2 (>|0.9) with no significant departure from linearity (P value = 0.1827), which indicates a strong linear relationship between the two variables. The slope is significantly different from zero (P value<0.0001) and the confidence interval for the slope is 95%.

Supporting Information

Figure S1 MCH1 has a replication pattern consistent with a single chromosome replicating at constant rate. The Cooper-Helmstetter model for DNA replication [76] predicts the DNA distribution in an ideal culture and replication parameters can be estimated from computer-simulations of the DNA histograms [16,77–78]. Cultures of V. cholerae, WT (left panels) or MCH1 (right panels), were grown exponentially with different carbon-sources to obtain independent samples with different cell-cycle parameters and samples were analyzed by flow cytometry. We compared the experimental DNA histograms obtained by flow cytometry to computer simulations of DNA contents in ideal cultures using the approach described by Michelsen et al [77]. The DNA histograms were simulated assuming either two chromosomes (WT) or one chromosome (MCH1) as described by [16]. In these simulations, the DNA histograms are resolved into the contributions from cells in the B, C and D periods. Shown are samples grown in M9+fructose (upper panels) and M9+fructose+serine (lower panels). Purple dots are actual DNA contents data, green curves simulate pre-replicating (B period) cells, blue curves simulate replicating (C period) cells, red curves simulate post-replicating (D period) cells and black curves accumulates the B, C and D period cells. The difference between the one and two chromosome simulations shows mainly in the shape of distribution of replicating cells: the increased replication rate late in the cell cycle with both chromosomes replicating lowers the blue C-curve compared to the same curve in cells with one chromosome. (TIF)

Figure S2 Construction of a mutant of V. cholerae, ESC1, with equally sized chromosomes. A. V. cholerae MV153 strain with attR/L sites from λ and HK022 phages inserted at precise loci. Recombination sites are located as follows: attRHK in the intergenic
region of [VC1939–VC1940] and attL in the integenic region of [VC901–VC902] on chrI; attHR in the integenic region of [VCA620–VCA629] and attR in the integenic region of [VCA514–VCA515] on chrII. B. Recombination [attR × attL] and [attHR × attHL] mediated by the expression of Int5 × Xis5 and Int5 × Xis5 HK. C. Recombination events [attR × attL] regenerate lacZ+ allowing for phenotypic detection of rearranged chromosomes. Recombination [attR × attL] and [attRHK × attLHK] leads to the transfer of 1 Mbp from chrI to chrII and the exchange of df1 and df2 sites. D. Edihium bromide stained pulse-field-gel electrophoresis of genomic DNA: lane 1, WT; lane 2, MCH1; lane 3, ESC1; lane 4, H. wingei marker (BioRad).

(TIF)

### Table S1
**Generation time of various genomic mutants in fast growing conditions.**

| Mutant         | Generation Time (min) |
|----------------|-----------------------|
| WT             | 1057                  |
| MCH1           | 2656                  |
| ESC1           | 3576                  |

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**Text S1** Supporting methods.

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**Author Contributions**

Conceived and designed the experiments: M-EV DM. Performed the experiments: M-EV OS MD-G. Analyzed the data: M-EV OS MD-G. Contributed reagents/materials/analysis tools: M-EV OS MD-G. Wrote the paper: M-EV DM.
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