Functionality of Two Origins of Replication in Vibrio cholerae Strains With a Single Chromosome

Matthias Bruhn¹, Daniel Schindler², Franziska S. Kemter¹, Michael R. Wiley³, Kitty Chase³, Galina I. Koroleva³, Gustavo Palacios³, Shanmuga Sozhamannan⁴,⁵* and Torsten Waldminghaus¹*

¹ LOEWE Centre for Synthetic Microbiology-SYNMIKRO, Philipps-Universität Marburg, Marburg, Germany, ² Manchester Institute of Biotechnology, The University of Manchester, Manchester, United Kingdom, ³ United States Army Medical Research Institute of Infectious Diseases, Frederick, MD, United States, ⁴ Defense Biological Product Assurance Office, Frederick, MD, United States, ⁵ The Tauri Group, LLC, Alexandria, VA, United States

Chromosomal inheritance in bacteria usually entails bidirectional replication of a single chromosome from a single origin into two copies and subsequent partitioning of one copy each into daughter cells upon cell division. However, the human pathogen Vibrio cholerae and other Vibrionaceae harbor two chromosomes, a large Chr1 and a small Chr2. Chr1 and Chr2 have different origins, an oriC-type origin and a P1 plasmid-type origin, respectively, driving the replication of respective chromosomes. Recently, we described naturally occurring exceptions to the two-chromosome rule of Vibrionaceae: i.e., Chr1 and Chr2 fused single chromosome V. cholerae strains, NSCV1 and NSCV2, in which both origins of replication are present. Using NSCV1 and NSCV2, here we tested whether two types of origins of replication can function simultaneously on the same chromosome or one or the other origin is silenced. We found that in NSCV1, both origins are active whereas in NSCV2 ori2 is silenced despite the fact that it is functional in an isolated context. The ori2 activity appears to be primarily determined by the copy number of the triggering site, crtS, which in turn is determined by its location with respect to ori1 and ori2 on the fused chromosome.

Keywords: DNA replication, secondary chromosome, plasmid, multipartite genome, replication initiation, pathogens, cholera

INTRODUCTION

The generally accepted paradigm of chromosome replication in bacteria is elucidated in Escherichia coli. Replication is initiated at a unique singular sequence, the origin of replication (oriC) by DnaA, proceeds bidirectionally along the chromosome and ends at the terminus diametrically opposite to oriC on the circular chromosome. In E. coli and related bacteria, immediate re-initiation of chromosome replication is hindered due to the hemi-methylated status of the sister chromosomes and sequestration of oriC by SeqA which has a high binding affinity to hemimethylated ori sequences (Lu et al., 1994; Slater et al., 1995; Waldminghaus and Skarstad, 2009). Most bacteria have single chromosomes and follow this general replication paradigm. However, about 10% of bacterial species have more than one chromosome and exhibit some deviation from this norm (Fournes et al., 2018). Among these, Vibrio cholerae with chromosome 1 (Chr1, ~3 Mbps) and
chromosome 2(Chr2, ∼1 Mbps) has served as a model system for studies pertaining to multi-chromosome replication mechanisms, and in recent years, an extensive body of information has been accumulated on various aspects of Chr1 and Chr2 replication (Egan et al., 2005; Iha et al., 2012; Val et al., 2014b; Espinosa et al., 2017; Ramachandran et al., 2017).

Chr1 in V. cholerae is similar to the E. coli chromosome in that the replication follows the same pattern: replication origin, ori1, contains multiple DnaA boxes, which are bound by DnaA that unwinds the DNA and initiate replication (Duigou et al., 2006). The similarity is so striking that V. cholerae ori1 can functionally substitute the E. coli replication origin oriC (Egan and Waldor, 2003; Demarre and Chattoraj, 2010; Koch et al., 2010; Kamp et al., 2013).

In contrast, the V. cholerae Chr2 appears to have an origin that resembles those of low copy number plasmids such as P1 and F (Fournes et al., 2018). The ori2 contains an array of repeats (iterons) where the Chr2 specific initiator protein, RctB, binds and unwinds the DNA for ori2 firing (Egan and Waldor, 2003; Duigou et al., 2008) but also exerts a form of negative regulation, termed ‘handcuffing,’ originally discovered in plasmids (Venkova-Canova and Chattoraj, 2011). Although ori2 has plasmid-like features, Chr2 resembles typical chromosomes in some respects: (1) Participation of SeqA and Dam in regulation of ori2 (Saint-Dic et al., 2008; Demarre and Chattoraj, 2010; Koch et al., 2010; Stokke et al., 2011). (2) Indispensability of Chr2, unlike plasmids, for cell survival because it harbors essential genes (Heidelberg et al., 2000; Kamp et al., 2013). (3) High level of coordination of replication between Chr1 and Chr2 in order to prevent over replication of Chr2 and ensure a guaranteed inheritance of a single copy of both chromosomes (Baek and Chattoraj, 2014; Val et al., 2016; Ramachandran et al., 2018). This raises the question on how coordination between Chr1 and Chr2 with respect to their timing of replication initiation is achieved given the disparity in their sizes and mechanisms of replication.

Chr1 replication is initiated at the onset of the replication period while initiation of Chr2 is delayed and occurs only when 2/3rd of Chr1 replication has been completed. Since Chr2 is 1/3rd the size of Chr1, both chromosomes consequently terminate their replication roughly at the same time (Rasmussen et al., 2007; Stokke et al., 2011). This termination synchrony appears not to be accidental but is selected for during evolution and is conserved within Vibrionaceae despite differing ratios of chromosome sizes (Kemter et al., 2018). This synchrony occurs through the crtS (Chr2 replication triggering Site) present on Chr1 that positively regulates ori2 initiation (Val et al., 2016). Translocation of the crtS locus on Chr1, either closer to ori1 or farther away, resulted in a corresponding shift in Chr2 initiation time as revealed by marker frequency analysis (Val et al., 2016), indicating that the native position of crtS sets the timing of Chr2 replication initiation such that its replication terminates synchronously with Chr1 (Kemter et al., 2018). The exact mechanism of crtS action remains to be elucidated but may include physical contacts between crtS and ori2 as well as sequestration of Chr2 replication initiator protein, RctB (Baek and Chattoraj, 2014; Val et al., 2016). Recently, the global transcription factor Lrp was shown to bind to the crtS site and to facilitate RctB binding (Ciaccia et al., 2018). Heterologous E. coli systems have been established based on ori2 mini-chromosomes demonstrating that crtS provided in trans increases mini chromosome copy number indicating a positive role played by crtS in ori2 firing (Baek and Chattoraj, 2014; Schallopp et al., 2017; de Lemos Martins et al., 2018). Recently, it was demonstrated that the copy number of crtS rather than the act of replicating the crtS is critical in the triggering of ori2 firing (de Lemos Martins et al., 2018; Ramachandran et al., 2018).

In order to assess the differential genetic requirements of Chr1 and Chr2 replication, an artificial single chromosome V. cholerae strain has been created by genetic engineering in which ori1 drives the replication of the fused chromosome (Val et al., 2012). In this strain, designated MCH1, the sequences to the left and right of ori2 were fused to the terminus of Chr1. In this arrangement the direction in which chromosome arms are replicated is conserved to minimize conflicts between DNA replication and transcription. The MCH1 strain was instrumental in establishing the essentiality of Dam methyltransferase in V. cholerae because of its role in ori2 function which was first shown by Demarre and Chattoraj (2010). This conclusion was further supported by the finding that depletion of Dam leads to spontaneous chromosomal fusion (Val et al., 2014a). In this case, the entire genome is replicated from ori1 which can tolerate the absence of Dam (Val et al., 2014a).

Recently, we described two naturally occurring V. cholerae strains (NSCV1 and NSCV2) containing both ori1 and ori2 on the same chromosome (Chapman et al., 2015; Xie et al., 2017). In these strains, Chr1 and Chr2 are fused at two different locations (Figure 1). The locations of relevant features such as ori1, ori2, and crtS sites are indicated in Figure 1A. In NSCV1, crtS is located about 670 kbs away from ori1 (Figure 1). It is similar to the standard two-chromosome reference strain N16961 with respect to distance, where crtS is located 695 kbs away from ori1. In NSCV2, the distance between ori1 and crtS is 1,566 kbs due to a large inversion that has occurred around the terminus region of the chromosome (Figure 1C).

This genomic architecture raised interesting questions about whether both origins are functional in the same cell or one or the other ori is silenced since in principle a single origin should suffice to replicate the fused chromosome. The strains also allowed us to ask if the chromosomal fusions are maintained without resorting to genome splitting which is the predominant genome configuration in Vibrionaceae. We found that in NSCV1 both origins are active in the same cell whereas in NSCV2 ori2 appears to be silent. Further, these chromosomes appear to be in a locked configuration since even after prolonged continuous growth they remain fused without splitting into two.

RESULTS

Activity of ori1 and ori2 in V. cholerae NSCV Strains

In general, the replication in bacteria relies on one origin of replication for one replicon. Fusion of two replicons, such as seen in NSCV1 and NSCV2, would initially give rise to a chromosome with two functional replication origins where one could be
superfluous. This raises the question of whether both, ori1 and ori2 are active in the fused chromosomes of NSCV1 and NSCV2, or only one of the origins is active and the other is silent. Inspection of the sequence of the origins and the replication initiator genes revealed no obvious mutational changes that could indicate non-functionality of one or the other of the origins in the two NSCV strains (Figure 2) (Xie et al., 2017). Compared to strain N16961, both strains possess complete ori1 sequences,
with seven SNPs (NSCV1) and four SNPs (NSCV2) spanning the 474 bps long gidA-mioC intergenic region and none of the DnaA boxes were affected by mutations. The 5,656 bps long ori2 regions (including genes parB, parA, and rctB) are also intact, with 54 SNPs in NSCV1 and 57 SNPs in NSCV2. Notably, the RctB-binding iteron sequences are not affected by mutations.

To test the activity of replication origins experimentally, we carried out marker frequency analyses (MFA). It is known that actively growing cells have a higher copy number of ori proximal sequences compared to ori distal/ter proximal sequences. We employed next generation sequencing technology to obtain whole genome sequences of DNAs isolated from stationary phase cultures of NSCV1 and NSCV2 and analyzed the sequence data for marker frequency. Read data from stationary phase DNAs were used for normalization and read mapping plots were created with ori1 repositioned at the center of the plot to represent bidirectional replication as well as for easy visualization of ori activity (Figure 3). Both NSCV1 and NSCV2 exhibited a maximum copy number of reads close to ori1 and a decreasing gradient on either side of ori1 moving toward the terminus creating a tent-shape (Skovgaard et al., 2011), indicating an active ori1 and bidirectional replication in both strains. In strain NSCV1, a local higher marker frequency was observed at the ori2 position, indicating that ori2 also is active in this strain. In contrast, no such local peak in marker frequency was found at the ori2 position in NSCV2 consistent with a silent ori2. In addition, an almost 3X lower ori1/ter ratio was observed in NSCV2 compared to NSCV1 indicating less or no overlap of replication cycles. The rationale for this interpretation is as follows: On a replicon with overlapping replication, the ori1/ter ratio would be four since replication is initiated twice before termination can occur. A replicon that initiates only once would consequently have an ori1/ter ratio of two. Considering that the culture represents a mixed population of cells before and after termination an ori1/ter ratio of higher than two, as in the case of NSCV1, indicates overlapping replication cycles while values less than two indicate no overlap of replication cycles.

**NSCV1 and NSCV2 Carry a Functional ori2**

The apparent inactivity of ori2 in strain NSCV2 raises the question of whether this origin of replication is functional but silenced or non-functional. To answer this question, we cloned the origins into a mini replicon and assessed independent replication in a plasmid backbone. Plasmid pMA135 carries oriR6K and can replicate conditionally in *E. coli* strains that provide in trans, the replication initiator protein, Pir, from a lambda prophage. In addition pMA135 can be transferred by conjugation from a donor to a recipient (Messerschmidt et al., 2015). In the absence of λpir, no exconjugants are obtained unless replication is driven by another fully functional origin of replication. The ori2 fragments of NSCV1 and NSCV2, including the core ori2 plus the genes rctB and parAB2, were cloned into pMA135 independently and the number of exconjugants was enumerated in an *E. coli* strain that does not contain λpir. The ori2 minichromosome constructs from all three strains (N16961, NSCV1, and NSCV2) yielded exconjugants at a frequency of about 1% (10^{-2}) of the recipients, as did the positive control F plasmid ori1 (Table 1). Minichromosomes based on oriII of strain N16961 have been shown not to integrate into the *E. coli* chromosome (Messerschmidt et al.,

---

### Table 1 | Conjugation efficiencies of replicons with NSCV ori2 replication origins.

| Replicon | Origin of replication | Conjugation efficiency |
|----------|-----------------------|-----------------------|
| pMA668   | N16961 ori2           | 5.5 × 10^{-3}         |
| pMA739   | NSCV1 ori2            | 1.7 × 10^{-2}         |
| pMA755   | NSCV2 ori2            | 4.3 × 10^{-2}         |
| pMA899   | F plasmid origin      | 3.2 × 10^{-2}         |
| pMA135   | oriR6K only           | 0                     |

All replicons possess an oriR6K and conjugated from *E. coli* strain WM3064 to MG1655. Efficiency of one representative experiment is given as ratio of conjugant CFU over total recipient CFU.

---

![FIGURE 3](https://example.com/figure3.png) | Marker frequency analysis (MFA) of *V. cholerae* NSCV1 (A) and NSCV2 (B) to assess origin activities. Profiles of genome-wide copy numbers based on Illumina sequencing and read mapping. Gray dots represent log numbers of normalized reads as mean values for 1 kbp windows relative to the stationary phase sample. The genome position is shown as the distance from ori1. Vertical dotted black lines mark the locations of replication origins and the crts site. The solid black lines represent the fitting of regression lines and the green line corresponds to the Loess regression (F = 0.05). Maxima are highlighted by red and minima as blue dots. Plots of biological replicates are shown in Supplementary Figure S1.
potentially could revert back to a two-chromosome arrangement upon prolonged continuous culturing. If splitting of the fused chromosomes occurs and if this splitting leads to a fitness advantage due to increased growth rates one would expect two-chromosome clones to appear in a population of NSCV1 and NSCV2 and these clones could potentially replace the fused chromosome cells after prolonged growth by clonal expansion. To test this hypothesis, we cultured the two NSCV strains for 16 days in 100 ml of liquid medium with replenishment of fresh medium every 24 h resulting in approximately 160 generations of growth in total. To examine if the NSCV strains reverted back to a two-chromosome arrangement we isolated DNA from long-term grown cells and performed long-read DNA sequencing using the PacBio technology to be able to detect long range chromosomal rearrangements such as the chromosomal fusion junctions/or junctions (~24–51 kbs) of the split chromosomes. A de novo genome assembly led to one single contig for both NSCV1 and NSCV2 reflecting the original one-chromosome configuration. In conclusion, the chromosome fusions in NSCV1 and NSCV2 appear to be stable and chromosome splitting is not a frequent event or the fused state is probably under positive selection pressure.

Genetic Stability of Chromosome Fusions in NSCV1 and NSCV2

An active ori2 in strain NSCV1 could potentially allow the chromosome fusion to be reversed. Similarly, the silent ori2 in NSCV2 might be inactive only in the context of a fused chromosome; in either case, splitting of Chr1 and Chr2 is conceivable. It was observed that V. cholerae with an artificially fused chromosome grew slower compared to the two-chromosome parental strain indicating a negative fitness burden on the bacterium (Val et al., 2012). Similarly, we observed an increased doubling time of strains NSCV1 (20 ± 0.5 min) and NSCV2 (29 ± 1.3 min) compared to the two-chromosome reference strain N16961 (16 ± 0.2 min) (Supplementary Table S4). In addition, microscopic examination showed that strain NSCV2 cells exhibited a distinct phenotype. While the two-chromosome reference strain N16961 and NSCV1 cells are comma-shaped as typical for V. cholerae, NSCV2 cells are much more curled and occasionally S-shaped (Figure 4). It is unknown whether this phenotype is related to the chromosome fusion.

It is conceivable that the slower growth of the fused chromosome strains may have negative fitness value leading to instability, thus promoting genome splitting. On the other hand, if the fused chromosome were under a positive selection pressure to remain fused even at a greater cost in terms of slower growth rate or if they are locked in the fusion configuration due to genetic defects that prevent splitting, then the fusion would be stable even after long term continuous culturing. This led us to test whether the two NSCV strains potentially could revert back to a two-chromosome arrangement upon prolonged continuous culturing. If splitting of the fused chromosomes occurs and if this splitting leads to a fitness advantage due to increased growth rates one would expect two-chromosome clones to appear in a population of NSCV1 and NSCV2 and these clones could potentially replace the fused chromosome cells after prolonged growth by clonal expansion. To test this hypothesis, we cultured the two NSCV strains for 16 days in 100 ml of liquid medium with replenishment of fresh medium every 24 h resulting in approximately 160 generations of growth in total. To examine if the NSCV strains reverted back to a two-chromosome arrangement we isolated DNA from long-term grown cells and performed long-read DNA sequencing using the PacBio technology to be able to detect long range chromosomal rearrangements such as the chromosomal fusion junctions/or junctions (~24–51 kbs) of the split chromosomes. A de novo genome assembly led to one single contig for both NSCV1 and NSCV2 reflecting the original one-chromosome configuration. In conclusion, the chromosome fusions in NSCV1 and NSCV2 appear to be stable and chromosome splitting is not a frequent event or the fused state is probably under positive selection pressure.

Replication Origins Are Active in an Engineered System Resembling the NSCV Arrangement

Why is ori2 of strain NSCV2 silenced while it is active in NSCV1? One obvious difference between the two strains is the differential positioning of the two replication origins ori1 and ori2 to one another. While the distance between ori1 and ori2 is 1.828 Mbps in NSCV1 it is only 1.118 Mbps in the genome of NSCV2.

To test experimentally, if the ori positioning of NSCV1 and NSCV2 influences the initiation outcome, we re-constructed the ori1 to ori2 arrangement in a genetically accessible system since NSCV1 and NSCV2 are recalcitrant for genetic manipulations. To accomplish this, we transferred a functional hapR to V. cholerae strain MCH1 to render it naturally competent (Lo Scrudato and Blokesch, 2012, 2013). Strain MCH1 was derived from the prototype V. cholerae strain N16961 by fusion of the two chromosomes with a deletion of ori2 (Val et al., 2012). We inserted a copy of ori2 including the flanking genes

![Figure 4](image-url)
parAB and rctB into MCH1 at positions analogous to NSCV1 and NSCV2 with respect to the distance from ori1 giving rise to strains VC61 and VC62, respectively, and performed marker frequency analysis of exponentially grown cultures (Figure 5).

The MFA plot of strain VC61 resembled the pattern seen in NSCV1 (Figure 5A, compared to Figure 3A). In contrast, a clear local peak of marker frequency was seen at the ori2 position of strain VC62 (Figure 5B). Thus, even though the origin arrangement is similar in NSCV2 and VC62, the ori2 copy appears to be active only in the engineered strain VC62 and not in NSCV2. To test if the ori2 positioning at the NSCV2 position has more negative effect on growth compared to the ori2 insertion at the NSCV1 position we measured the doubling times of strains VC61 (26 ± 0.1 min) and VC62 (27 ± 0.5 min) (Supplementary Table S4). The difference in doubling time was only marginally affected suggesting that the differences in DNA replication between the two strains do not result in severe impairment in growth. We conclude that an ori2 insertion at positions analogous to those in the NSCV strains relative to ori1 can be active as determined by MFA.

**Dam Methyltransferase Is Functional in NSCV Strains**

We have shown above that ori2 in strain NSCV2 is functional but appears to be not active. One potential cause of ori2 silencing could be an inactive Dam methylation system. Methylation of the adenine within the Dam recognition site ‘GATC’ present at ori2 locus is a prerequisite for ori2 activity (Demarre and Chattoraj, 2010). Sequence analyses showed intact dam genes in both strains (Xie et al., 2017). The methylation status of GATC sites can be analyzed by the differential sensitivities of the genomic DNA to DpnI (cleaves only methylated/hemimethylated GATC sites), DpnII (cleaves only unmethylated GATC sites) and Sau3A1 (cleaves both unmethylated and methylated GATC sites) restriction enzymes.
Genomic DNAs of NSCV1 and NSCV2 were sensitive to DpnI and Sau3A1 and resistant to DpnII, indicative of full methylation at GATC sites (Figure 6). This result was further confirmed by the PacBio sequence data. In PacBio SMRT (Single Molecule Real Time) sequencing, presence of modified base (A in GATC) in the DNA template results in a delayed incorporation of the corresponding T nucleotide, i.e., longer inter-pulse duration (IPD) compared to template lacking the modification (Flusberg et al., 2010). These kinetic measurements create specific signatures for different types of base modifications. Analyses of the PacBio sequence data for modified bases confirmed by the PacBio sequence data. In PacBio SMRT sequencing, we extracted the respective sequences and aligned them to the consensus sequence we established recently (Figure 7A) (Kemter et al., 2018). Notably, all highly conserved parts of the crtS sequence are also conserved in the crtS sequences of NSCV1 and NSCV2 (Figure 7A). To test the functionality of crtS in the context of a fused chromosome experimentally, we deleted the crtS in strain VC62 (resulting in strain VC71) and performed marker frequency analysis (Figure 7B).

As expected, no local peak of copy number increase was seen at the ori2 position of the ΔcrtS strain VC71 in contrast to the MFA of the parental strain VC62 (compare Figures 5B, 7B) confirming the necessity of a functional crtS site for ori2 firing. Incidentally, the doubling time of this strain is not much different (25 ± 0.6 min) from that of VC61 and VC62 (Supplementary Table S4). To analyze the functionality of the crtS sites from NSCV1 and NSCV2, we inserted these sequences into the genome of E. coli and transformed the corresponding strains with the ori2-based minichromosome synVicII. The rationale behind this approach is the observation of a copy number increase of an ori2-based minichromosome in E. coli strains carrying a functional crtS site (Baek and Chatteraj, 2014; Kemter et al., 2018). The copy number of ori2 minichromosomes was measured in E. coli strains either carrying the crtS sites of strain N16961, NSCV1, NSCV2 or no crtS (Figure 7C). In this case, we tested the resistance level of the respective strains to ampicillin as an indirect measure of the minichromosome copy number as described previously (Schallop et al., 2017). The crtS sites of both NSCV strains increased the copy number of the ori2 minichromosome compared to the strain lacking any crtS site and to a similar extent as the crtS site from the two-chromosome V. cholerae strain N16961 used as positive control (Figure 7C). We conclude that crtS sites in NSCV1 and NSCV2 are functional in a heterologous system and a defective crtS might not explain the silent ori2 in strain NSCV2.

**Relative ori1, ori2, and crtS Locations Determine ori2 Activity in NSCV Strains**

In a canonical two-chromosome V. cholerae, replication of the crtS on Chr1 triggers the initiation at ori2 on Chr2. This scenario can in principle be analogous in a fused chromosome such as NSCV1 in which ori1, ori2 and crtS are present on the same molecule. Here the crtS is located about 677 kbps away from ori1 and is replicated first with replication forks originating at ori1 which subsequently triggers ori2 firing, which is further downstream of crtS. The organization is different in strain NSCV2 where the crtS lies about 416 kbps downstream of ori2. Thus, in NSCV2, ori2 precedes crtS. This arrangement is the consequence of a large inversion of the Chr1 part of the genome (Figure 1). The replicative outcome of this arrangement is difficult to predict considering the known triggering role of crtS; i.e., ori2 being replicated first by replication forks originating at ori1, subsequently upon duplication of the crtS site. In other words, crtS can also retrospectively trigger replication of already replicated ori2 that crtS replication is supposed to trigger in the...
first place. To test this scenario, we moved the crtS site in strain VC62 to the position analogous to strain NSCV2 (resulting in strain VC73). VC73 did not exhibit any significant difference in doubling time compared to other strains (26 ± 0.2 min) (Supplementary Table S4). Respective MFA analysis revealed an active ori2, suggesting that an ori2 copy on a fused chromosome can be triggered by a crtS site located downstream relative to ori2, contrary to what was observed in NSCV2 (compare Figures 3B–8). The peak at the ori2 position is not very strong but it is important to note that the fitting of regression lines is automated based on the maxima found within the mapped reads (Kemter et al., 2018). There is no pre-selection of ori positions and the fact that the peak is detected at the ori2 position by the computational fitting model implies that it is an active origin as also seen in the biological replicate (Supplementary Figure S1F).

DISCUSSION

*Vibrio cholerae* has partitioned its genome between a true bacterial chromosome and a “domesticated” plasmid replicon (Heidelberg et al., 2000; Venkova-Canova and Chattoraj, 2011; Val et al., 2014b). Unlike most bacteria, this two-replicon arrangement is conserved within the family of *Vibrionaceae* (Okada et al., 2005; Val et al., 2014b; Ramachandran et al., 2017). It was postulated that the bipartite genome in *Vibrio* species enables varying the copy numbers of both chromosomes in a niche-specific manner under certain environmental conditions as an adaptation strategy (Heidelberg et al., 2000; Schoolnik and Yildiz, 2000; Srivastava and Chattoraj, 2007). Alternatively, the two-chromosome setup in *Vibrio* species can be considered as an adaptive feature that enables rapid genome duplication if multiple chromosomes are replicated simultaneously. The latter assumption fits with the observation that *Vibrio* species is one of the fastest growing bacteria known, a feature that has led to a recent proposal for using the non-pathogenic *V. natriegens* as the new workhorse for bioengineering (Weinstock et al., 2016; Dalia et al., 2017; Hoffart et al., 2017). However, it brings up the question of how the two replications are coordinating their replication and regulate over or under replication in order to ensure inheritance of the just one copy of the full complement of the genome into daughter cells upon cell division. Furthermore, the evolutionary driving
force (or forces) that led to the two-chromosome setup in *Vibrionaceae* remain speculative. One way to better understand the evolutionary significance of this unique feature of *Vibrio* species is to study naturally occurring exceptions to the two chromosome rule: strains which have evolved into a single chromosome by chromosomal fusion. We have previously identified two Natural Single Chromosome *Vibrio* (NSCV) *cholerae* strains (Chapman et al., 2015; Johnson et al., 2015; Xie et al., 2017) and in this study we addressed the functionality and activity of the two origins of replication on the same chromosome.

Fortuitously, the two NSCV strains have different fusion junctions and other genetic rearrangements that provided an opportunity to compare and contrast their ori functionality. We used marker frequency analysis as an indirect measure of ori activity and found ori1 to be active in both strains. In strain NSCV1, we detected an additional, replication activity peak at the ori2 locus indicating that this second origin is active as well. Since the MFA experiments are population based, we cannot exclude that some cells use ori1 only and other cells ori2 only to replicate the fused chromosome. However, we consider it more likely that both origins are firing within the same cell. Interestingly, the copy number ratio of ori2/ter was lower than for ori1/ter consistent with a time delayed initiation of ori2 on the fused chromosome. Such an origin initiation differential has also been observed in conventional two-chromosome *Vibrio* strains resulting in synchronous termination of replication of the two chromosomes despite their different sizes (Rasmussen et al., 2007; Stokke et al., 2011; Kemter et al., 2018). Conservation of the orchestrated replication timing of the two origins in NSCV1 indicates that the single-chromosome setup does not lead to any origin interference. In two-chromosome *Vibrio* species, replication of the *crtS* site located on Chr1 has been shown to trigger ori2 firing (Baek and Chattoraj, 2014; Val et al., 2016; Ramachandran et al., 2018). In *V. cholerae* N16961, the time between *crtS* replication and ori2 initiation corresponds to the time the replication fork needs to replicate about 200 kbps of DNA (Val et al., 2016). Considering a similar delay in strain NSCV1, ori2 would have fired long before replication fork originating at ori1 reaches it since the distance between *crtS* to ori2 is more than 1 Mbps. As a consequence, replication forks originating at ori1 and ori2 will meet at some position of the fused chromosome, the timing of which will depend on ori1 location and its regulation just as it occurs in two-chromosome *V. cholerae*.

The scenario is much different in NSCV2. Here, ori2 is replicated before *crtS* by replication forks originating at ori1 (Figure 1A). Intuitively, this would lead to a chaotic replication perturbation because ori2 firing precedes *crtS* duplication. In the two-chromosome context, *crtS* duplication occurs first which then triggers ori2 firing. Hence, if replication of the *crtS* triggers ori2 initiation also in this genomic arrangement it would happen on two copies of the already replicated ori2. In addition, the replication forks coming from this newly initiated ori2 copies have the potential to replicate the *crtS* in just a few minutes because of their close proximity and could potentially lead to additional rounds of ori2 initiation and thus an uncontrolled ori2 firing. If this were to happen, not only replication control at ori2 would be severely perturbed but also ori1 functioning can be interfered because ori1 might be replicated passively from replication forks, coming from ori2. Interestingly, what we observed in strain NSCV2 is not a replication out of control but instead a simple silencing of ori2 activity. Our data clearly demonstrate that ori2 of NSCV2 is functional in an isolated context as shown by its ability to drive replication of a mini-chromosome in *E. coli*. In addition, critical factors involved in regulation of ori2 appear to be fully functional in NSCV2, namely the Dam methylation system and the *crtS* site. Paradoxically, ori2 is not used to initiate DNA replication in the fused chromosome strain, NSCV2.

A recent study offers a simple explanation for our observation on ori2 silencing in NSCV2 (Ramachandran et al., 2018). These authors showed that it is the doubling of the *crtS* dosage rather than the process of replicating the *crtS* that triggers ori2 initiation which results in an even number of *crtS* and ori2 copies. This tendency of the regulatory system to produce similar copy numbers of *crtS* and ori2 have also been found in engineered systems with multiple *crtS* sites (de Lemos Martins et al., 2018). If the *crtS* site is duplicated before ori2 as it is the case in two-chromosome *V. cholerae* strains, the ori2 copy number will be lower compared to *crtS* and consequently the initiation at ori2 will be triggered to restore *crtS*/ori2 copy number balance. If the *crtS* site is replicated after ori2 has been copied by replication forks coming from ori1 as in the case of NSCV2, there is no need to initiate at ori2 because the copy number of *crtS* and ori2 are in balance already. The lack of initiation at ori2 in NSCV2 is therefore fully consistent with and confirmatory to the findings of the aforementioned studies (de Lemos Martins et al., 2018; Ramachandran et al., 2018). However, in contrast to the expectation of the *crtS*-to-ori2 copy control model, we observed that ori2 is active in the engineered strain VC73 in which the *crtS* lies downstream of ori2 analogous to the arrangement in NSCV2 (Figure 8). However, it is important to note that although the positioning of ori1, ori2 and *crtS* are similar in strains NSCV2 and VC73, the genomic contexts of ori2 and *crtS* are entirely artificial compared to their native positions and furthermore, VC73 does not share the large chromosomal inversion found in NSCV2. More precisely, in VC73, ori2 lies in a region of the original Chr1 and *crtS* within a region of the original Chr2 (Figure 8, left panel). The discrepancy in ori2 firing between the naturally occurring NSCV2 and the engineered strain VC73 may therefore be explained by their different genomic context.

One interesting question that arises is if the two *V. cholerae* strains with fused chromosomes are evolutionarily stable. It could also be that the fusion occurred only transiently as an artifact of lab cultivation or during the original strain isolation from a patient sample. In fact, chromosome fusions in *V. cholerae* have been observed previously to occur frequently within a population but the two chromosome configuration seems to provide a selective advantage leading to rapid elimination of the fused chromosomes cells from the population (Val et al., 2014a). Based on this observation, we grew the NSCV strains for about 160 generations and expected a splitting of the
fused chromosome that may provide a selective advantage thereby eliminating the cells with single chromosomes from the population. Contrary to the expectation, we found that cells retained the fused chromosomes indicating that they are in fact, locked in this configuration and not easily revertible. An alternative explanation would be that the fused state is under positive selection pressure. It remains to be seen what the significance of the fused chromosome status in NSCV1 and NSCV2 is, in contrast to vast majority of other V. cholerae strains where two chromosome status is the norm. In any case, the single-chromosome V. cholerae appears to be more frequent than expected as yet another NSCV strain was discovered recently (Yamamoto et al., 2018). We expect future studies of NSCV strains which are a deviation from the norm, would lead to a better understanding of why most V. cholerae carry their genome split into two as the norm.

**MATERIALS AND METHODS**

**Strains, Plasmids, Oligonucleotides, and Growth Conditions**

All strains, plasmids and oligonucleotides used in this study are listed in the Supplementary Tables S1–S3. Unless indicated otherwise, the bacterial cells were grown in LB medium at a temperature of 37°C. Antibiotic selection was performed at the following concentrations, if not indicated differently: Ampicillin 100 μg/ml, Kanamycin 35 μg/ml for E. coli and 70 μg/ml for V. cholerae, Spectinomycin 100 μg/ml, Gentamicin 20 μg/ml, Chloramphenicol 35 μg/ml. Where needed, dianminopimelic acid (DAP) was added to the medium at a concentration of 300 μM. To determine doubling times, cells were grown in LB medium in a 96-well plate at 37°C in a microplate reader (Infinite M200 pro multimode microplate reader, Tecan). OD

600 was measured every 5 min for 18 h. Doubling times were calculated in exponential phase for OD

600 values between 0.01 and 0.1. For continues cultivation of NSCV strains to investigate potential chromosome splitting strains were inoculated in the morning and grown in 100 ml liquid medium for 24 h. The next morning, new flasks were inoculated 1:1,000 from the previous cultures. After 4 working days (on day 5), 1 ml samples of the cultures were frozen at −80°C as glycerin stocks. Two days later, new flasks were inoculated from these glycerin stocks and the procedure was continued for a total of 16 days. Before sequencing, these were re-streaked on TCBS medium to verify that it is V. cholerae and to obtain single colonies. For each strain one single colony was used to inoculate a culture for DNA isolation and sequencing.

**Construction of Replicons and Strains**

NSCV minichromosomes were constructed by PCR amplifying the respective origins of replication using the primers 1227/1228 on V. cholerae NSCV1 genomic DNA for pMA739 or V. cholerae NSCV2 genomic DNA for pMA755. The vector pMA135 was digested with Ascl and the PCR products were integrated in the linearized vector using Gibson Assembly (Gibson et al., 2009) used to transform E. coli WM3064. For construction of chromosomal integrations and deletions, integration cassettes were assembled by using the MoClo system as described previously (Weber et al., 2011; Milbredt et al., 2016; Schindler et al., 2016). The ori2 insertion cassettes on pMA735 and pMA736, as well as the crtS deletion and insertion cassettes on pMA748 and pMA749 were assembled by MoClo reactions using the plasmids indicated in Supplementary Table S2, which themselves were assembled by MoClo reactions of the respective PCR products into the respective backbones (primers, templates, and backbones indicated in Supplementary Table S2). The linear cassettes were released by restriction enzyme digestions with Bsal. Triparental mating was performed to deliver the plasmids pUXBF13 and pGP704-mTn7-hapR_ATN from E. coli S17-1 λpir to V. cholerae MCH1 (Bao et al., 1991; Melbom et al., 2005; Val et al., 2012). The created strain V. cholerae VC49 was naturally
and transferred to *E. coli* described (Schindler et al., 2016) and used to transform products were assembled in pMA349 by MoClo assembly as primers 1439/1440 from gDNA of plasmid pMA449, *crtS* cassette from pMA748 and a flippase reaction to excise the *V. cholerae* and VC62 were created (De Souza Silva and Blokesch, 2010). transformation with pBR-flp released from pMA735 or pMA736, respectively, followed by competent and by transforming it with the ori2 insertion cassettes released from pMA735 or pMA736, respectively, followed by transformation with pBR-flp and flippase reaction strains VC61 and VC62 were created (De Souza Silva and Blokesch, 2010). *V. cholerae* strain VC71 was constructed by deletion of the *crtS* sequence by transforming *V. cholerae* VC49 with the *crtS* deletion cassette from pMA748 and a flippase reaction to excise the resistance marker. Subsequently, strain VC71 was transformed with the *crtS* insertion cassette from pMA749 and the flippase reaction was performed to create strain VC73. To construct plasmid pMA449, *crtS* was amplified with primers 1439/1440 from gDNA of *V. cholerae* NSCV1 and for pMA450 with primers 1439/1440 from gDNA of *V. cholerae* NSCV2. PCR products were assembled in pMA349 by MoClo assembly as described (Schindler et al., 2016) and used to transform *E. coli* TOP10 cells. Details of further MoClo assemblies are provided in Supplementary Table S2. Assemblies were used to transform *E. coli* DH5α λpir. The derived integration cassettes were cut out with BsaI, integrated into the chromosome of *E. coli* AB330 and transferred to *E. coli* MG1655 per P1-transduction. FRT recombination was used to remove the resistance marker.

**Sequence Comparison**

Sequences from *V. cholerae* N16961, NSCV1 and NSCV2 were compared by multiple sequence alignment using Clustal Omega (Goujon et al., 2010; Sievers et al., 2011) to find single nucleotide polymorphisms. To analyze the whole genomes of the *V. cholerae* strains NSCV1 and NSCV2 for sequence homology, the alignment free chromosome comparison tool SMASH (Pratas et al., 2015) was used with a minimum block size setting of 5,000 bp.

**Preparation of Genomic DNA From Bacteria**

The desired amount of culture (between 0.1 ml and 5 ml) was mixed 1:1 with ice-cold killing buffer and centrifuged at maximum speed and 4°C for 3 min. The pellet was resuspended in 300 µl TE, 40 µl SDS and 3 µl 0.5 M EDTA. After 5 min incubation at 65°C, 750 µl Isopropanol were added and the sample was centrifuged 5 min at maximum speed. The pellet was resuspended in 500 µl TE and 2 µl RNase were added. After 30 min at 37°C, 2 µl of Proteinase K were added and incubated for another 15 min. The sample was purified via twofold Phenol-Chloroform extraction and precipitated over night at −20°C by mixing it with 1 ml pure ethanol and 40 µl 3 M sodium acetate.

The next day, DNA was spun down at maximum speed for 10 min, the pellet was washed with 70% ethanol and centrifuged another 10 min. The pellet was dried and resuspended in 50 µl pure water.

**Short Read Sequencing (Illumina MiSeq)**

*V. cholerae* genomic DNA was isolated as described (Kemter et al., 2018) from various strains grown under different conditions (log phase vs. stationary) were quantitated using the Qubit fluorometer (Thermo Fisher) and adjusted to 0.2 ng/µL with nuclease-free water. Sequencing libraries were prepared using the Illumina Nextera XT kit, processed and pooled according to manufacturer's instructions (Illumina). The final, pooled sample was paired-end sequenced (2 x 300 bp) using the Illumina MiSeq with a v3 chemistry, 600 cycle kit. Post sequencing processing was performed the systems software packages and the final demultiplexed fastq reads produced by the instrument were used for MFA against the reference genome. Raw sequencing data are available on request.

**Long Read Sequencing (PacBio)**

Whole genome sequencing was performed on a Pacific Biosciences RSII platform. The sequencing library was prepared using the SMRTbell™ Template Prep Kit (Pacific Biosciences, Menlo Park, CA, United States) following manufacturer's protocol. 5 µg of DNA was fragmented using gTUBE (Covaris Inc., Woburn, MA, United States) to ~20 kb. After DNA damage repair and ends repair, blunt hairpin adapters were ligated to the template. Non-ligated products were digested by ExoIII and ExoVII exonuclease. Resulting SMRTbell template were purified with AMPure PB beads and size selected on BluePippin system (Sage Science, Beverly, MA, United States), using 0.75% dye-free agarose cassette, with 4–10 kb Hi-Pass protocol and lower cut set on 4 kb. Size selected purified libraries were quantified by Qubit dsDNA High Sensitivity assay. After primer annealing, and P6 polymerase binding, templates were bound to MagBeads for loading. Each sample was sequenced on two SMRT cells, using C4 sequencing kit and 360-min movies per SMRT cell. Presence of unidentified contaminant in two libraries (NSV2 16 days) inhibited sequencing reactions, which manifested in extremely low P1 (2%) and super short reads. Both libraries were subjected to the cleanup procedure that involves binding annealed SMRTbell libraries to magnetic beads, washing the bound annealed DNA SMRTbell templates to remove potential contaminant, and eluting the purified, annealed DNA SMRTbell templates from the magnetic beads. The purified SMRTbell templates were then re-quantified by Qubit and prepared for sequencing on the PacBio RSII according to the Binding Calculator. After cleanup procedure, PacBio RS II instrument sequencing yields were comparable to the other samples. Raw sequencing data are available on request.

**Marker Frequency Analysis**

Sequencing reads from a NGS were mapped to the respective genome using the program Geneious (Biomatters Ltd.; Kearse et al., 2012). Read densities were extracted and plotted using custom R scripts as described previously (Kemter et al., 2018).

**Semiquantitative Conjugation**

All used replicons possess an oriR6K and were conjugated from *E. coli strain* WM3064 to MG1655. For overnight cultures of donor and recipient strains the OD600 was determined and the amount of cells corresponding to 1 ml of OD600 = 1 was centrifuged 1 min at 13,000 x g. The cells were washed twice in TBS and resuspended in 100 µl TBS. From each donor strain, 50 µl were mixed with 50 µl of the recipient strain and dropped on LB agar including DAP. After 6 h cells were scraped off the plate, washed twice in TBS. The total CFU of recipient cells was
determined by plating dilutions on LB, while the CFU of plasmid bearing recipients was determined by plating the same dilutions on selective media. The selective CFU was then normalized to total CFU.

Microscopy
Differential interference contrast (DIC) microscopy was performed on 1% (w/v) agarose pads in PBS buffer using the Nikon Ti fluorescence microscope (100× objective, NA 1.45).

Quantification of Replicon Copy Number via Antibiotic Sensitivity
The copy-up effect of 
<sup>crtS</sup>
via Antibiotic Sensitivity was measured as described (Messerschmidt et al., 2016). Cells were grown in LB medium with either 100 or 500 µg/ml ampicillin at 37°C in 96-well plates in a microplate reader (Infinite M200 pro multimode microplate reader, Tecan). The main culture (150 µL) was inoculated 1:1,000 and growth curves recorded for 15 h. For better visualization, 1 divided by the time needed to reach an OD<sub>600</sub> of 0.1 was defined as measure of the copy number.

AUTHOR CONTRIBUTIONS
MB, DS, SS, and TW contributed to the conceptualization of the study. MB, DS, FK, MW, KC, GK, and GP contributed to the methodology. MB, DS, KC, and GK contributed to the software. MB, DS, FK, MW, KC, GK, SS, and TW contributed to the formal analysis of the study. MB, SS, and TW contributed to the writing of the original draft. SS and TW reviewed and edited the manuscript. DS, SS, and TW provided the supervision. SS, GP, and TW contributed to the funding acquisition.

FUNDING
This work was supported within the LOEWE program of the State of Hesse and a grant of the Deutsche Forschungsgemeinschaft (Grant No. WA2713/4-1). Funding for the sequencing work was provided by National Strategic Research Institute (NSRI, University of Nebraska) under the contract number FA4600-12-D-9000 – TOPR 0042 – TO0059).

ACKNOWLEDGMENTS
We thank all current and former members of the Waldminghaus lab for help and fruitful discussions. Nadine Schallopp is acknowledged for excellent technical assistance and Mehryad Mataei for experimental support. We are grateful to Melanie Blokesch for advice in 
<em>V. cholerae</em> genetics and for providing strains and plasmids and to Didier Mazel for providing 
<em>V. cholerae</em> strain MCH1.

SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02932/full#supplementary-material
Messerschmidt, S. J., Schindler, D., Zumkeller, C. M., Kemter, F. S., Schallopp, N., Gibson, D. G., Young, L., Chuang, R. Y., Venter, J. C., Hutchison, C. A., Bruhn et al. (2015). Two Replication Origins on a Single Chromosome. Biochim. Biophys. Acta 1819, 826–829. doi: 10.1016/j.bbagrm.2012.01.012

Johnson, S. L., Khan, A., Bishop-Lilly, C. A., Chapman, C., Patel, M., Verratti, K., Jha, J. K., Baek, J. H., Venkova-Canova, T., and Chattoraj, D. K. (2017). A transcriptional regulator modulator of replication initiation in E. coli. Cell 1747, 826–829. doi: 10.1016/j.cell.2018.10.026

Kemter, F. S., Kemter, F. S., and Waldminghaus, T. (2016). Optimization and characterization of the synVicII and Waldminghaus, T. (2016). Chromosome dynamics in multichromosome bacteria. Biochim. Biophys. Acta 1819, 826–829. doi: 10.1016/j.bbagrm.2012.01.012

Lo Scrudato, M., and Blokesch, M. (2013). A transcriptional regulator linking quorum sensing and chitin induction to render Vibrio cholerae naturally transformable. Nucleic Acids Res. 41, 3644–3658. doi: 10.1093/nar/gkt041

Lu, M., Campbell, J. L., Boye, E., and Kleckner, N. (1994). SeqA: a negative modulator of replication initiation in E. coli. Cell 77, 413–426. doi: 10.1016/0092-8674(94)90156-2

Meibom, K. L., Blokesch, M., Dolganov, N. A., Wu, C. Y., and Schoolnik, G. K. (2005). Chitin induces natural competence in Vibrio cholerae. Science 310, 1822–1827. doi: 10.1126/science.1120696

Messerschmidt, S. J., Kemter, F. S., Schindler, D., and Waldminghaus, T. (2015). Synthetic secondary chromosmes in Escherichia coli based on the replication origin of chromosome II in Vibrio cholerae. Biotechnol. J. 10, 302–314. doi: 10.1002/biot.201400031

Messerschmidt, S. J., Schindler, D., Zumkeller, C. M., Kemter, F. S., Schallopp, N., and Waldminghaus, T. (2016). Optimization and characterization of the synthetic secondary chromosome synVicII in Escherichia coli. Front. Bioeng. Biotechnol. 4:96. doi: 10.3389/fbioe.2016.00096

Milbredt, S., Farmani, N., Sobetzko, P., and Waldminghaus, T. (2016). DNA replication in engineered Escherichia coli genomes with extra replication origins. ACS Synth. Biol. 5, 1167–1176. doi: 10.1021/acssynbio.6b00064

Okada, K., Iida, T., Kita-Tsukamoto, K., and Honda, T. (2005). Vibrios commonly possess two chromosomes. J. Bacteriol. 187, 752-757. doi: 10.1128/JB.187.7.752-757.2005

Pratas, D., Silva, R. M., Pinho, A. J., and Ferreira, P. J. (2015). An alignment-free method to find and visualise rearrangements between pairs of DNA sequences. Sci. Rep. 5:10203. doi: 10.1038/srep10203

Ramachandran, R., Ciaccia, P. N., Flores, T. A., Jha, J. K., and Chattoraj, D. K. (2018). Chromosome 1 licenses chromosome 2 replication in Vibrio cholerae by doubling the crsT gene dosage. PLoS Genet. 14:e1007426. doi: 10.1371/journal.pgen.1007426

Ramachandran, R., Jha, J., Paulsson, J., and Chattoraj, D. (2017). Random versus cell cycle-regulated replication initiation in bacteria: insights from studying Vibrio cholerae chromosome 2. Microbiol. Mol. Biol. Rev. 81, e00033-16

Ramussen, T., Jensen, R. B., and Skovgaard, O. (2007). The two chromosomes of Vibrio cholerae are initiated at different time points in the cell cycle. EMBO J. 26, 3124–3131. doi: 10.1038/sj.emboj.7601747

Saint-Dic, D., Kehrli, J., Frushour, B., and Kahng, L. S. (2008). Excess SeqA leads to replication arrest and a cell division defect in Vibrio cholerae. J. Bacteriol. 190, 5870–5878. doi: 10.1128/JB.00479-08

Schallopp, N., Milbredt, S., Sperle, T., Kemter, F. S., Bruhn, M., Schindler, D., et al. (2017). Establishing a system for testing replication inhibition of the Vibrio cholerae secondary chromosome in Escherichia coli. Antibiotics 7:3.E3. doi: 10.3390/antibiotics7010003

Schindler, D., Milbredt, S., Sperle, T., and Waldminghaus, T. (2016). Design and assembly of DNA sequence libraries for chromosomal insertion in bacteria based on a set of modified MoClo vectors. ACS Synth. Biol. 5, 1362–1368. doi: 10.1021/acssynbio.6b00089

Schoolnik, G. K., and Yildiz, F. H. (2000). The complete genome sequence of Vibrio cholerae: a tale of two chromosomes and of two lifestyles. Genome Biol. 1, 1016.1–1016.3. doi: 10.1186/gb-2000-1-3-reviews 1016

Sievers, F., Wilm, A., Dineen, D., Gibson, T. J., Karplus, K., Li, W., et al. (2011). Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol. Syst. Biol. 7:339. doi: 10.1038/msb.2011.75

Skovgaard, O., Bak, M., Lobner-Olesen, A., and Tommerup, N. (2011). Genome-wide detection of chromosomal rearrangements, indels, and mutations in circular chromosomes by short read sequencing. Genome Res. 21, 1388–1393. doi: 10.1101/gr.117416.110

Slater, S., Wold, S., Lu, M., Boye, E., Skovgaard, O., and Kleckner, N. (1995). E. coli SeqA protein binds oriC in two different methyl-modulated reactions appropriate to its roles in DNA replication initiation and origin sequestration. Cell 82, 927–936. doi: 10.1016/0092-8674(95)90272-4

Srivastava, P., and Chattoraj, D. K. (2007). Selective chromosome amplification in Vibrio cholerae. Mol. Microbiol. 66, 1016–1028. doi: 10.1111/j.1365-2958.2007.05973.x

Stokke, C., Waldminghaus, T., and Skarstad, K. (2011). Replication patterns and organization of replication forks in Vibrio cholerae. Microbiology 157, 695–708. doi: 10.1099/mic.0.045112-0

Val, M. E., Kennedy, S. P., Soler-Bistue, A. J., Barbe, V., Bouchier, C., Ducos-Galand, M., et al. (2014a). Fuse or die: how to survive the loss of Dam in Vibrio cholerae. Mol. Microbiol. 91, 665–678. doi: 10.1111/mmi.12483

Val, M. E., Soler-Bistue, A., Bland, M. J., and Mazel, D. (2014b). Management of multipartite genomes: the Vibrio cholerae model. Curr. Opin. Microbiol. 22, 120–126. doi: 10.1016/j.mib.2014.10.003

Val, M. E., Marbouty, M., de Lemos Martins, F., Kennedy, S. P., Kemble, H., Bland, M. J., et al. (2016). A checkout control orchestrates the replication of the two chromosomes of Vibrio cholerae. Sci. Adv. 2:e1501914. doi: 10.1126/sciadv.1501914

Val, M. E., Skovgaard, O., Ducos-Galand, M., Bland, M. J., and Mazel, D. (2012). Genome engineering in Vibrio cholerae: a feasible approach to address biological issues. PLoS Genet. 8:e1002472. doi: 10.1371/journal.pgen.1002472
Venkova-Canova, T., and Chattoraj, D. K. (2011). Transition from a plasmid to a chromosomal mode of replication entails additional regulators. *Proc. Natl. Acad. Sci. U.S.A.* 108, 6199–6204. doi: 10.1073/pnas.1013244108

Waldminghaus, T., and Skarstad, K. (2009). The *Escherichia coli* SeqA protein. *Plasmid* 61, 141–150. doi: 10.1016/j.plasmid.2009.02.004

Weber, E., Engler, C., Gruetzner, R., Werner, S., and Marillonnet, S. (2011). A modular cloning system for standardized assembly of multigene constructs. *PLoS One* 6:e16765. doi: 10.1371/journal.pone.0016765

Weinstock, M. T., Hesek, E. D., Wilson, C. M., and Gibson, D. G. (2016). *Vibrio natriegens* as a fast-growing host for molecular biology. *Nat. Methods* 13, 849–851. doi: 10.1038/nmeth.3970

Xie, G., Johnson, S. L., Davenport, K. W., Rajavel, M., Waldminghaus, T., Detter, J. C., et al. (2017). Exception to the rule: Genomic characterization of naturally occurring unusual *Vibrio cholerae* strains with a single chromosome. *Int. J. Genomics* 2017:8724304. doi: 10.1155/2017/8724304

Yamamoto, S., Lee, K. I., Morita, M., Arakawa, E., Izumiya, H., and Ohnishi, M. (2018). Single circular chromosome identified from the genome sequence of the *Vibrio cholerae* O1 bv. El Tor Ogawa strain V060002. *Genome Announc.* 6:e00564-18. doi: 10.1128/genomeA.00564-18

**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Bruhn, Schindler, Kemter, Wiley, Chase, Koroleva, Palacios, Sozhamannan and Waldminghaus. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.