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Minireview

Exception to the exception rule: synthetic and naturally occurring single chromosome *Vibrio cholerae*

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Summary

The genome of *Vibrio cholerae*, the etiological agent of cholera, is an exception to the single chromosome rule found in the vast majority of bacteria and has its genome partitioned between two unequally sized chromosomes. This unusual two-chromosome arrangement in *V. cholerae* has sparked considerable research interest since its discovery. It was demonstrated that the two chromosomes could be fused by deliberate genome engineering or forced to fuse spontaneously by blocking the replication of Chr2, the secondary chromosome. Recently, natural isolates of *V. cholerae* with chromosomal fusion have been found. Here, we summarize the pertinent findings on this exception to the exception rule and discuss the potential utility of single-chromosome *V. cholerae* to address fundamental questions on chromosome biology in general and DNA replication in particular.

Exception to the rule: two chromosome *V. cholerae*

Beginning in 1989, there was a paradigm shift in the idea that eukaryotes are exclusive in possessing multiple chromosomes. The seminal discovery of multipartite chromosomal architecture in bacteria was revealed by pulse-field gel electrophoresis (PFGE) results of *Rhodobacter sphaeroides* (Suwanto and Kaplan, 1989). Since then many other bacteria have been shown to have divided genomes of circular and/or linear nature (diCenzo and Finan, 2017). These include *Agrobacterium tumefaciens*, *Burkholderia cepacia* complex and many more (Allardet-Servent *et al*., 1993; Rodley *et al*., 1995; Egan *et al*., 2005). The presence of two chromosomes in *V. cholerae* was revealed by PFGE and later confirmed by whole-genome sequencing (Trucksis *et al*., 1998; Heidelberg *et al*., 2000). In fact, it was demonstrated early on that all the tested species (39) in *Vibrionaceae* possess two chromosomes (Okada *et al*., 2005). With the explosion in bacterial whole-genome sequences using next-generation sequencing approaches in the last decade, many more bacteria with multipartite genomes have been uncovered: among the 16 328 complete prokaryotic genomes, 980 strains (~6.0%) have been listed as having more than one chromosome encompassing ~375 unique species spanning different bacterial phyla (NCBI, 2020). While in some phylogenetic groups only a subset of species/strains appear to have multiple chromosomes (e.g. *Erwinia amylovora* ATCC49946 versus strain LA635) in other families and genera such as *Vibrionaceae*, *Brucella* and *Burkholderia*, it appears to be the norm (diCenzo and Finan, 2017; NCBI, 2020) Most of our knowledge on the control of chromosome replication, maintenance and segregation of multipartite genomes is derived from studies on *V. cholerae* (Egan *et al*., 2005; Jha *et al*., 2012; Val *et al*., 2014a).

It was hypothesized that the Chr2 of *V. cholerae* originated from a plasmid and evolved into a secondary chromosome by adding additional layers of regulation for its replication (Heidelberg *et al*., 2000; Venkova-Canova and Chattoraj, 2011). Although Chr1 encodes the majority of the housekeeping genes and is considered as the main chromosome, Chr2 also harbours essential genes and many genes with unknown functions (Cameron *et al*., 2008; Chao *et al*., 2013; Kamp *et al*., 2013). Functional biases seen in the genes distributed between the large

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and the small chromosomes of *V. cholerae* and in other bacteria with divided genomes provide an explanation on how the occupation of specialized niches by different bacteria may have driven the evolution of the multipartite genomes (Schoolnik and Yildiz, 2000; diCenzo and Finan, 2017). Niche-specific differential expression of the genes on Chr1 and Chr2 in *V. cholerae* has been observed. For example, when the bacterium was grown mid-exponentially in rabbit ileal loops, it showed expression of many more genes of Chr2 than those expressed in aerobically grown cells in rich medium and harvested at the mid-exponential phase (Xu *et al*., 2003). Majority of these are probably important niche-specific genes and hence expressed preferentially in ileal loop and stool. Similar results were seen when the bacteria were collected from the stools of cholera patients (Merrell *et al*., 2002).

In a landmark study, Egan *et al*. defined the boundaries of the origins of replications of the primary (Chr1) and the secondary chromosome (Chr2) of *V. cholerae* (Egan and Waldor, 2003). Chr1 replication follows the traditional *Escherichia coli* paradigm in that the replication origin (*ori1*) contains *cis* specific elements such as the DnaA boxes, acted on in a programmed manner by *trans* acting factors such as DnaA, the replication initiator protein and other accessory factors such as DNA adenine methyltransferase (Dam) (Egan and Waldor, 2003; Duigou *et al*., 2006; Demarre and Chattoraj, 2010). The replication origin of Chr2 (*ori2*) resembles that of low copy number plasmids such as P1 and F in that it contains an array of repeats (*iterons*), which are bound in a sequence-specific manner by the Chr2-specific initiator protein, RctB, to unwind the DNA at *ori2* to initiate replication (Egan and Waldor, 2003; Duigou *et al*., 2006; Duigou *et al*., 2008; Gerding *et al*., 2015). In addition, unlike plasmids, there are additional layers of regulation that enable Chr2 to function like *bona fide* chromosomes: (i) more stringent replication control than plasmids; (ii) sharing some of the Chr1 accessory replication factors; (iii) timing and coordination of replication initiation, termination and segregation with Chr1 (Egan and Waldor, 2003; Venkova-Canova and Chattoraj, 2011; Jha *et al*., 2012; Val *et al*., 2014a). Recent studies have advanced our understanding of the replication initiation asynchrony and termination synchrony. In *V. cholerae*, it appears that Chr1 and Chr2 initiate replication at different times in the cell cycle but terminate at the same time (Rasmussen *et al*., 2007; Stokke *et al*., 2011). How do the chromosomes talk to each other to coordinate replication and cell cycle? The signal for the smaller Chr2 to start replication initiation comes from the *crtS* (*C*hr2 *r*eplication *t*riggering *S*ite), which is located about 850 kbps downstream of *ori1* on Chr1 (Val *et al*., 2016). Replication of the *crtS* triggers initiation of DNA replication at *ori2* on Chr2 by a not yet fully understood mechanism. Further support for the role of *crtS* comes from studying the genomic location of *crtS* sites on Chr1 relative to the size of Chr2 in different *Vibrio* species; i.e. a corresponding shift in triggering time of *ori2* replication initiation with an increase in Chr2 size to coincide with termination and thus evolutionary conservation of this phenomenon (Kemter *et al*., 2018). Additional interesting findings towards the understanding of the underlying mechanism are the binding of the *ori2* initiator RctB as well as the protein Lrp to *crtS* and its spatial association with *ori2* (Baek and Chattoraj, 2014; Val *et al*., 2016; Ciaccia *et al*., 2018) and the fact that *crtS* not only regulates the timing of Chr2 replication initiation but also controls Chr2 copy number (de Lemos Martins *et al*., 2018; Ramachandran *et al*., 2018).

In addition to these sophisticated mechanisms of coordinated DNA replication, the presence of two chromosomes has led to the evolution of distinct partitioning mechanisms. The two chromosomes of *V. cholerae* are longitudinally arranged in the cell (David *et al*., 2014). While Chr1 appears to be spread along the entire longitudinal axis of the cell, Chr2 is restricted to the younger half of the cell. In newborn cells, Chr1 extends from the old pole to the new pole and Chr2 extends from midcell to the new pole (David *et al*., 2014). Each of the two *V. cholerae* chromosomes encodes its own specific partitioning system, namely ParAB1 and ParAB2, which recognize distinct sites exclusively carried on their respective chromosomes (Yamaichi *et al*., 2007a; Yamaichi *et al*., 2007b).

Exception to the exception rule: synthetic single-chromosome *Vibrio cholerae*

The facts that *V. cholerae* has two chromosomes as opposed to the normal single chromosome paradigm found in many bacteria and that both chromosomes are essential for viability of the bacterium raise questions on the evolutionary significance of divided genomes and the consequences of having a single fused chromosome. An obvious experimental approach is to create a fusion of the two natural chromosomes and study the consequences. This requires, on the one hand, a careful consideration of the genomic architecture of an ideal fusion chromosome and, on the other hand, sophisticated genetic tools that could be brought to bear to construct such a fusion. Both challenges were tackled by the group of Didier Mazel who successfully constructed the first synthetic single-chromosome *V. cholerae* (Val *et al*., 2012). In their fusion design, these investigators kept the canonical organizational features of bacterial chromosomes (Hendrickson and Lawrence, 2006; Rocha, 2008). More specifically, the chromosomal fusion was designed to conserve the origin-to-terminus symmetry, gene

synteny, strand bias and polarities of the original replichores (Val *et al*., 2012). This was realized by fusing regions to the left and right of *ori2* to the terminus region of Chr1 (Fig. 1-top panel-right).

How was this chromosomal fusion accomplished? Val and colleagues (2012) employed site-specific recombination systems. Such genetic tools have been used before to generate large chromosomal rearrangements (Medberry *et al*., 1995; Esnault *et al*., 2007). Val and colleagues (2012) improved upon this idea by using two different recombinases with their respective recognition sites. Notably, these site-specific recombination systems have the advantage of directional manipulation; i.e. they allow unidirectional fusion but prevent reversion of engineered fusion strains into their components. The two-recombinase method enabled the necessary simultaneous formation of specific fusion junctions in one step. The resultant MonoCHromosomal *V. cholerae* (MCH1) strain was viable and exhibited just a slightly increased generation time of 29 min compared with the parent strain (23 min). The MCH1 strain was useful to decipher many intriguing aspects of multi-chromosome replication. Val and colleagues (2012) started with the investigation of the essentiality of Dam (DNA adenine methyltransferase), which was found earlier to be essential for the wild type two-chromosome *V. cholerae* unlike in *E. coli*. Dam methylates the adenine nucleotide of the DNA sequence motif GATC and plays a role in many cellular functions such as *ori1*/*ori2* replication initiation, mismatch repair and gene expression (Lobner-Olesen *et al*., 2005). In the engineered single-chromosome *V. cholerae*, MCH1, all the *ori2* replication-associated genes could be deleted since replication of the entire chromosome is driven by *ori1*, thereby rendering Chr2 replication functions dispensable. The single chromosome study also substantiated the prediction that the

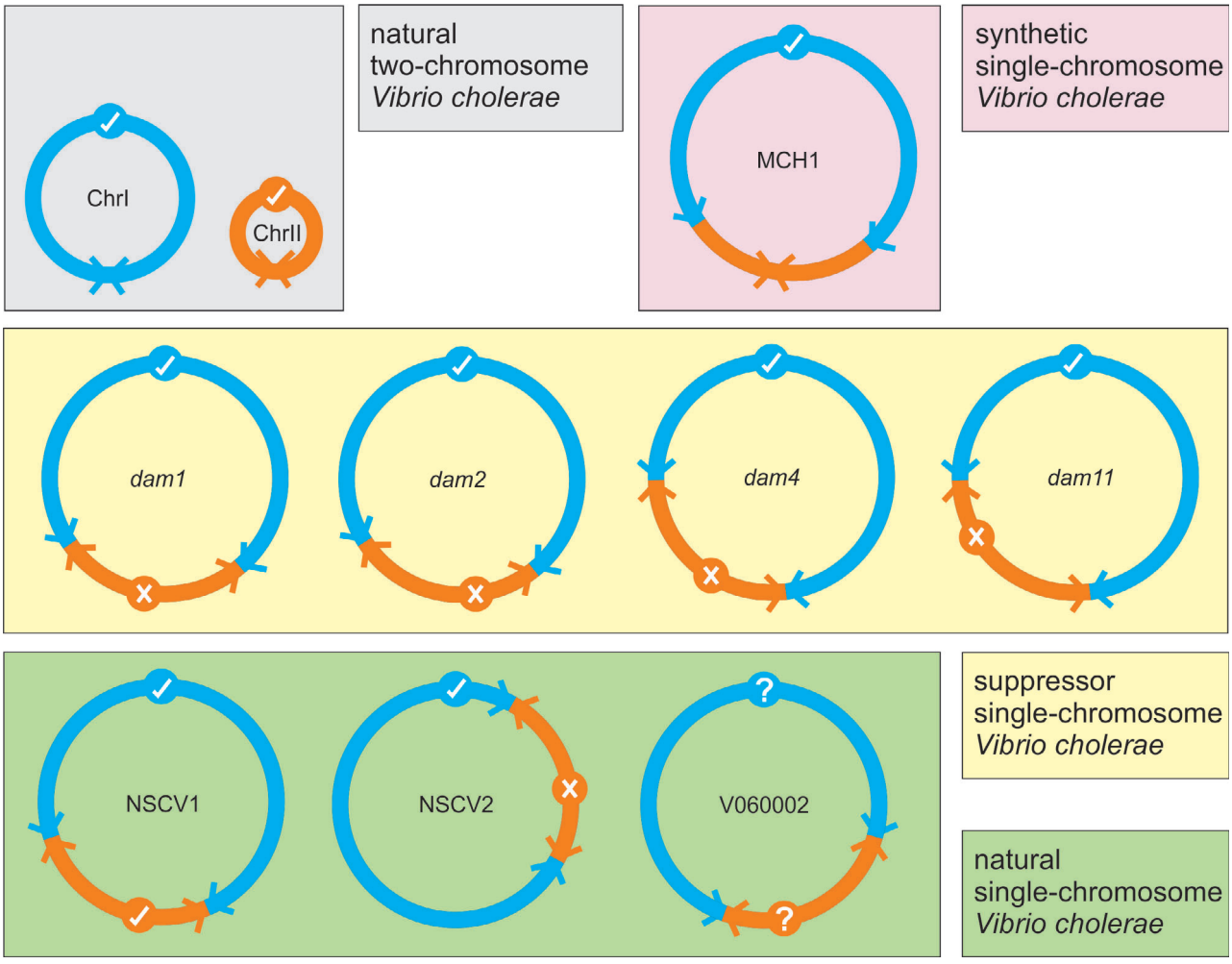

Fig. 1. Schematics of *V. cholerae* chromosomal fusion structures. Genome structures of one synthetic, four spontaneous suppressor mutants and three natural isolates with their respective origins of replications (dots) and original replication direction (arrows) are depicted. Origins are indicated by tick marks if functional, by crosses if non-functional and question marks if the functionality is not known. [Color figure can be viewed at wileyonlinelibrary.com]

essentiality of Dam resides primarily in its role in Chr2 replication as described below.

If a factor such as Dam is essential in wild type *V. cholerae* only because of its role in Chr2 replication, one way to bypass this requirement would be a chromosomal fusion. In the absence of Dam function (*dam* mutant), a Chr1-Chr2 fusion would allow replication of the genetic information encoded on Chr2 by *ori1* thereby making *ori2*-based replication obsolete. This is exactly what Val and co-workers observed as reported in their article titled 'Fuse or die: how to survive the loss of Dam in *Vibrio cholerae*' (Val *et al*., 2014b). The Dam protein was shown to be essential in wild type *V. cholerae* because the target DNA motif of the Chr2 replication initiator protein, RctB, includes a GATC (Dam enzyme recognition site) that needs to be in a methylated state to permit efficient binding and there are multiple RctB binding sites within *ori2* (Julio *et al*., 2001; Demarre and Chattoraj, 2010). Thus, deletion of *dam* was only possible in the presence of a complementing plasmid providing Dam protein *in trans* (Demarre and Chattoraj, 2010). By creating conditional lethality and forcing the complementing *dam* plasmid to be lost, Val and colleagues (2014b) were able to select suppressor strains. The frequency of these suppressors was higher than what is expected for spontaneous point mutations. Further analyses of the suppressor mutants by PFGE and whole-genome sequencing confirmed the prediction that the two chromosomes had been fused spontaneously (Val *et al*., 2014b). Interestingly, two different mechanisms were found to mediate such spontaneous chromosomal fusions: (i) homologous recombination between IS elements; (ii) site-specific recombination between the two *dif* sites (Fig. 1-middle panel). The *dif* sites reside in the terminus region of bacterial chromosomes and are used to resolve chromosomal dimers, which are formed by crossovers between sister chromatids (Lesterlin *et al*., 2004). The site-specific recombinases XerC and XerD act on the *dif* sites of Chr1 and Chr2 of *V. cholerae* although the respective *dif*-site sequences vary (Val *et al*., 2008). *Dif*-mediated site-specific recombination between Chr1 and Chr2 *dif* sites might occur frequently in natural *V. cholerae* populations but such events are probably counterselected due to selection pressure against fused chromosomes (Val *et al*., 2014b).

Although the biological, functional and evolutionary significance of a multipartite genome in bacteria are still less well understood, it has been suggested that multipartite organization is clearly stable and selected for, especially in *Vibrio* species (Val *et al*., 2014a). We have seen that Dam is essential only in wild type *V. cholerae* but not if chromosomes are fused because of its role in Chr2 replication. The same is true for the Chr2 initiator protein RctB and is expected to be true for any key factor associated with Chr2 replication. This assumption was further fortified by the selection of suppressor mutations that could rescue a deletion of the Chr2 triggering site *crtS* turned out to be chromosome fusions (Val *et al*., 2016). These findings point to a potential experimental approach to identify as yet unknown factors involved in Chr2 replication by comparing the set of essential genes of a wild type two-chromosome *V. cholerae* with a fused chromosome strain. Any gene being essential in the two-chromosome system but not in the chromosome-fusion strain would be an interesting candidate as a Chr2-specific replication factor. It is possible to identify Chr1-specific replication factors using the same strategy with a fused strain with only a functional *ori2* provided such an engineered strain is viable.

Exception to the exception rule: naturally occurring single-chromosome *Vibrio cholerae*

The general rule that all natural *V. cholerae* strains have two-chromosomes has been challenged by the discovery of two isolates of *V. cholerae*, formally designated as Natural Single Chromosome Vibrio (NSCV), in which the two chromosomes are fused (Chapman *et al*., 2015; Johnson *et al*., 2015). The two single-chromosome *V. cholerae* strains NSCV-1 [1154-74 (serogroup O49)] and NSCV-2 [10432-62 (serogroup O27)] were isolated in India and the Philippines respectively, several decades ago, from clinical samples of patients exhibiting non-cholera like diarrhoeal symptoms (Shimada *et al*., 1994). Accordingly, these strains apparently lack the typical cholera virulence factors such as CTX and TCP although other toxin genes are encoded in their genomes (Xie *et al*., 2017). Interestingly, in contrast to the findings of Val and colleagues (2012)) described above, the fusion of the two chromosomes in the two natural isolates seemed to have occurred via different motifs: more specifically fusions did not occur at the *dif* sites although site-specific or homologous recombination-mediated by repeats, IS elements or prophages are implicated (Xie *et al*., 2017) (Fig. 1-bottom panel). In addition, the genome fusions have occurred at different locations of Chr1 and Chr2 in the two strains and the *dam* gene is intact. In NSCV1 and NSCV2, both origins of replication are present and intact (Xie *et al*., 2017). The question of whether two types of origins of replication can function simultaneously on the same chromosome or one or the other origin is silenced was tested using next-generation sequencing-based marker frequency analyses. It was 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 (Bruhn *et al*., 2018; Bhabatosh and Dhruba, 2019). 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 (Bruhn *et al*., 2018). It is noteworthy that there is a large inversion in NSCV2 that shifts the position of *crtS* further away from *ori1* for an as yet undeciphered reason (Bruhn *et al*., 2018).

Additional instance of single chromosome exception

NSCV1 and NSCV2 are not the only exceptions to the two-chromosome rule in *V*. *cholerae*. Recently, whole-genome sequence of another single chromosome *V*. *cholerae* strain, V060002, was reported (Yamamoto *et al*., 2018). Unlike NSCV1 and NSCV2 that are non-cholera *Vibrios*, V060002 belongs to the pathogenic, cholera causing O1 biovar El Tor Ogawa group. It was isolated in 1997 from a patient who travelled to Indonesia and it has been in extensive use as a laboratory strain for genetic manipulations and studies pertaining to natural competence in *V*. *cholerae* (Yamamoto *et al*., 2014; Blokesch, 2016; Yamamoto and Ohnishi, 2017; Yamamoto *et al*., 2018; Dubnau and Blokesch, 2019). Comparative genome analyses of V060002 indicated that the Chr1 and Chr2 fusion has occurred between positions 1937484-1938781 of Chr1 and positions 735763-737020 of Chr2. The fused chromosome appears to have been generated by recombination between highly homologous insertion sequence elements shared by Chr1 and Chr2 (99% identity, corresponding to vc1789 to vc1790 on Chr1 and vca0791 to vca0792 on Chr2 of N16961) (Yamamoto *et al*., 2018). The authors further observed that these recombination sites are identical to those of a representative chromosomal fusion spontaneously isolated from N16961 with a null mutation of the *dam* gene (Val *et al*., 2014b), which is essential for Chr2 replication (Demarre and Chattoraj, 2010) (Fig. 1). Curiously, the *dam* gene of V060002 is identical to the canonical sequence found in a prototypical O1 strain N16961 (unpublished observation). Apparently, V060002 maintains the single chromosome status very stably and grows slower than typical two chromosome strains. It was also found that V060002 can support a plasmid carrying *ori2*, indicating that at least the replication initiator RctB is active in this strain (Shouji Yamamoto personal communication). Further characterization of this strain with respect to the functionality of the two origins by experiments such as marker frequency analyses might reveal whether both origins are active simultaneously in the same cell or not and how chromosome fusion occurred even with an intact wild type *dam* gene and finally, how the single chromosome status is stably maintained. Screening additional *V*. *cholerae* or other *Vibrio species* strains might uncover more unusual *Vibrios* with single chromosome that will undoubtedly expand our knowledge on the mechanisms of replication in chromosomes with multiple origins of replication.

What can we learn from single-chromosome *V*. *cholerae*?

Although it seems counterintuitive, as established in the foregoing narrative, studying single-chromosome *V*. *cholerae* can teach us more about two-chromosome bacterial replication. For example, the synthetically engineered single-chromosome *V*. *cholerae* strains were instrumental in demonstrating the essentiality of certain proteins linked to Chr2 replication (Val *et al*., 2012). Interestingly, a deletion of *dam* in the single-chromosome strain, although not lethal, reduced the viability substantiating its known role outside of *ori2*. Yet another result from this study was the experimental evidence for increased chromosomal dimer formation with increased replicon size (Val *et al*., 2012). Comparison of the synthetic single-chromosome *V*. *cholerae* to the ones selected as suppressors and the natural isolates reveals the astonishing flexibility of chromosomes with respect to extreme rearrangements. Chromosomes are generally thought of as well-organized rigid structures (Kepes *et al*., 2012; Sobetzko *et al*., 2012). It is well known that many genes are oriented in the direction of replication to avoid head-on collision of the transcription complex with the replication apparatus (Fig. 2) (Liu and Alberts, 1995; Mirkin and Mirkin, 2005). The same orientation bias was observed for chromosome maintenance motifs as for example FtsK-orienting polar sequences (KOPS) (Bigot *et al*., 2005; Sobetzko *et al*., 2016). In addition, replication of chromosomal regions in the reverse direction would be inhibited by termination systems such as Tus/ter in *E*. *coli* (Bussiere and Bastia, 1999) (Fig. 2). For the synthetic single-chromosome *V*. *cholerae* MCH1 such an organization was deliberately maintained in the original design of the construct (Val *et al*., 2012). However, the chromosomal fusions found in the natural single chromosome *V*. *cholerae* appear to challenge these rules (Fig. 2) (Chapman *et al*., 2015; Johnson *et al*., 2015). A functional terminus-to-terminus fusion does, for example exclude the existence of a termination system on Chr2 because the respective chromosomal regions are successfully replicated in reverse direction (Fig. 2) (Val *et al*., 2014b; Bruhn *et al*., 2018).

It is likely that chromosomal fusions occur frequently in a population of *Vibrio* cells and we hypothesize that such variants with genomic rearrangements are not evolutionarily fixed because of reduced fitness of these fusion variants. If fusion variants do not revert back by excision into two chromosome status, they probably are competed out by the wild type two chromosome cells. A fusion-excision event (reversion) could restore the original genomic state if excision occurs at the same sites as the fusion. Alternatively, this event could redefine the genetic content and size of the two chromosomes if excision occurs at sites

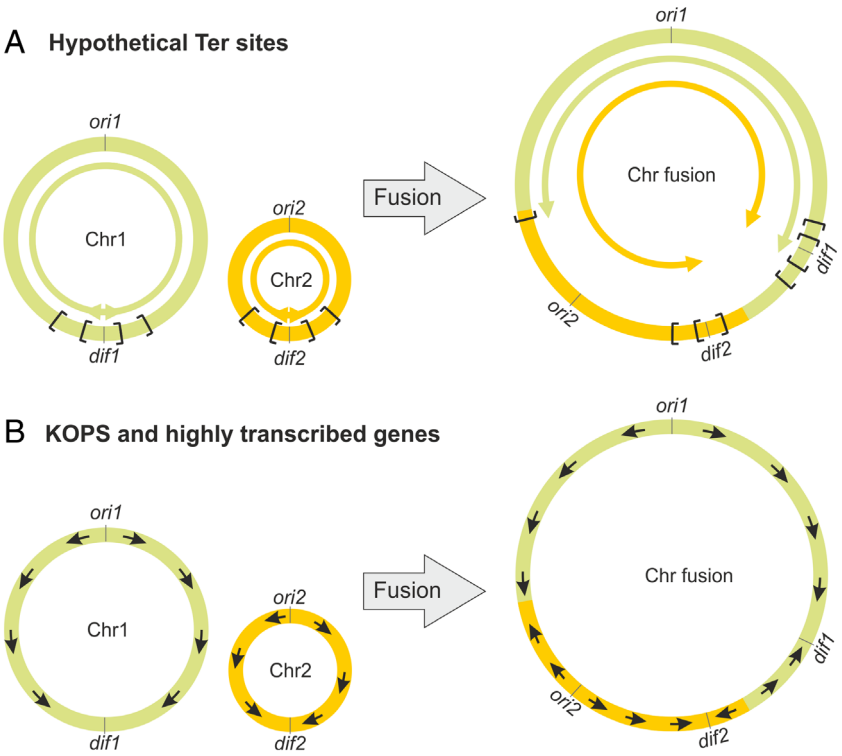

Fig. 2. Replication specific features of individual chromosomes and the consequences of chromosomal fusion. A. Ter sites (brackets) allow passage of the replication machinery in one direction only and consequently block replication in the reverse direction. In the fusion chromosome, the inner circles show how far replication forks originating at *ori1* (green) and *ori2* (orange) respectively would be able to move before they are stalled if there is a functional termination system in *V. cholerae*. Some regions of the chromosome would not be replicated if this were the case leading to cell death. The fact that Chr1 and Chr2 fusions are viable indicates that a functional termination system is absent in *V. cholerae*. B. FtsK-orienting polar sequences (KOPS) and highly transcribed genes (both shown as black arrows) are usually organized in *ori* to *ter* direction. Chromosomal fusions would perturb this arrangement. [Color figure can be viewed at wileyonlinelibrary.com]

different from the original fusion sites. To what extend such fusion-excision events shaped the genomic content and size of the two *Vibrio* chromosomes during the course of evolution is unknown at this time but it is evident that frequent genetic exchanges between the two chromosomes occur (Lukjancenko and Ussery, 2014).

Chromosome-sized DNA fragments have been fused into bacterial genomes before but one might expect that nature would select against such rearrangements (Itaya *et al*., 2005). The unusual natural *V. cholerae* isolates provide a unique opportunity to study the evolutionary adaptation of a fused chromosome. In this regard, one specific question of interest to be addressed is: Is it important for the chromosome to have most genes oriented in '*ori-ter*' direction? If so, a natural chromosomal fusion that inverts the orientation of the genes to '*ter-ori*' direction, would lead to an inversion of the orientation of those genes over time unless there is a selective pressure to preserve such a configuration or the fusion event happened recently and hence has not had enough evolutionary time to invert the genes to '*ori-ter*' orientation. Notably, an *E. coli* strain with the replication origin moved to an ectopic site led to a large chromosomal inversion to restore the replication-consistent gene orientation (Ivanova *et al*., 2015).

For many years, the city of Berlin was divided into Eastern and Western Berlin with each sub-city having its own civic infrastructures such as university, zoo and public transport system, and so forth. Post-cold war, the peaceful unification of Eastern and Western Berlin, raised questions on the need for parallel systems. Drawing from this analogy, this is probably what the single chromosome *V. cholerae* was forced to 'consider' because the two chromosomes had evolved two sets of independent and parallel features. More specifically, this question pertains to the two different replication and segregation systems. This is because NSCV strains do not follow all rules generally accepted for bacterial genomic architecture. One such rule is that bacteria have a single active replication origin on a single chromosome while eukaryotic organisms replicate their multiple chromosomes with multiple replication origins per chromosome (Kuzminov, 2014). Silencing one of the systems in NSCV strains makes economic and regulatory sense considering a coordinated regulation of replication and segregation; having two systems might interfere or cross-talk with each other when located on the same replicon. We are only beginning to understand the DNA replication and chromosome segregation in naturally occurring single chromosome *V. cholerae* strains and hence these strains are treasure troves to be explored to address these fundamental questions.

Synthetic biology approaches can pave the way for basic research findings

The pioneering work of Ron Breaker on synthetic RNAs that could bind chemical ligands with high affinity and specificity (Breaker and Joyce, 1994; Breaker, 1996) led

to the discovery of natural systems in which gene expression is regulated by ligand-induced structural changes in mRNAs (Winkler and Breaker, 2005). Comparative genomics and companion experiments led to the discovery of riboswitches and a growing number of new examples in synthetic biology are continuously being invented (Nahvi *et al*., 2002; Furukawa *et al*., 2015). We have narrated above a short historical perspective of an exception to the exception rule of two chromosome genome, beginning with synthetically engineered single-chromosome *V. cholerae* to their discovery in nature. In fact, many more examples of synthetic biology approaches have been undertaken to answer or raise fundamental questions on genome structure. This includes the construction of a linear *E. coli* chromosome (Cui *et al*., 2007), splitting of chromosomes onto two replicons (Itaya and Tanaka, 1997; Liang *et al*., 2013), and addition of extra replicons with chromosomal origins (Lobner-Olesen *et al*., 1987; Messerschmidt *et al*., 2015). The dawn of synthetic genome biology has provided an excellent avenue to push the boundaries further to investigate chromosome biology to its fullest potential (Annaluru *et al*., 2015; Schindler and Waldminghaus, 2015). Many chromosome maintenance systems that ensure the faithful segregation and retention of bacterial chromosomes have been described (Touzain *et al*., 2011; Messerschmidt and Waldminghaus, 2014). We envision that the design and construction of synthetic chromosomes will have a greater impact on understanding chromosome maintenance systems in the future.

Conclusions

The recent novel findings on synthetic and natural single chromosome *V. cholerae* reviewed here have opened up new avenues of research interest. Some questions to be addressed in future studies are: What is the evolutionary, functional and mechanistic significance of having two chromosomes as opposed to a single chromosome? What are the consequences of fusing the two chromosomes and how does it happen in nature and what is the frequency of its occurrence in nature? Once a chromosomal fusion occurs, how is it maintained and what are the genetic factors that enforce the unidirectional event and prevent the reversal into two chromosomes? Also, what are the factors that prevent chromosomes from merging in all strains with multipartite genome? More interestingly, the three natural single chromosome *V. cholerae* strains apparently harbour both origins of replication (*ori1* and *ori2*) and they are intact. At least in NSCV1 both origins are active simultaneously and this is the first example of a naturally existing bacterial chromosome with two functional replication origins although this idea has been proposed based on computational analyses of genome sequences (Gao, 2015) and also by genetically engineering an *E. coli* strain with multiple functional origins of replication (Wang *et al*., 2011; Milbredt *et al*., 2016). It is noteworthy that the experimentally generated *V. cholerae* chromosome fusions were all replicated from a single replication origin, *ori1* (Val *et al*., 2012; Val *et al*., 2014b). Since NSCV1 and NSCV2 are recalcitrant to genetic manipulations, synthetic biology approaches may aid in recapitulating the genomic structures of NSCV1 and NSCV2 in a different genetic background so that some of these mysteries can be solved. Alternatively, screening more natural *V. cholerae* isolates may unravel new single chromosome strains. The fact that *V. cholerae* does not exhibit the replication fork trap similar to *E. coli* (Galli *et al*., 2019) increases the likelihood of higher prevalence of single chromosome than realized (~2%) from our screening. We anticipate that future studies on natural and synthetic chromosome fusions in *V. cholerae* will undoubtedly shed new light on hitherto unknown mechanisms on all aspects of bacterial chromosome biology. They might also prove to be useful in developing *ori2*-specific antimicrobial substances which are expected to be lethal to two-chromosome but not to single-chromosome *Vibrios* (Schallopp *et al*., 2017).

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