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Genomic Plasticity of *Vibrio cholerae*

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Summary. *Vibrio cholerae* is one of the deadliest pathogens in the history of humankind. It is the causative agent of cholera, a disease characterized by a profuse and watery diarrhoea that still today causes 95.000 deaths worldwide every year. *V. cholerae* is a free living marine organism that interacts with and infects a variety of organisms, from amoeba to humans, including insects and crustaceans. The complexity of the lifestyle and ecology of *V. cholerae* suggests a high genetic and phenotypic plasticity. In this review, we will focus on two peculiar genomic features that enhance genetic plasticity in this bacterium: the division of its genome in two different chromosomes and the presence of the superintegron, a gene capture device that acts as a large, low-cost memory of adaptive functions, allowing *V. cholerae* to adapt rapidly.

Keywords: *Vibrio cholerae* · genome plasticity · superintegron

Introduction

Vibrio cholerae is a halophilic Gram-negative organism endemic to certain regions of Asia, such as the Ganges Delta. It is the causative agent of cholera, an infamous disease that produces a profuse watery diarrhoea with high mortality rates if untreated [25]. It is commonly found in saline coastal waters and estuaries, either as a free-living organism or forming biofilms on the chitinous surface of crustaceans [59,104]. This association to zooplankton seems central to its lifestyle, since chitin is the major carbon and nitrogen source for *V. cholerae*, as well as the signal that triggers a state of natural competence enabling extensive horizontal gene transfer (HGT) events [80]. Apart from the human intestine, *V.*

cholerae can infect or colonise a variety of distant organisms, including yeast [9,10], amoeba [1,86], flies [15], and mice [47]. Such distant habitats highlight the adaptability of *V. cholerae* and are suggestive of a remarkable underlying genetic plasticity. Indeed, the arsenal of genetic weapons that *V. cholerae* uses to thrive during infection varies depending on the host: for instance, the main virulence factors in humans and some animal models are the cholera toxin [81], encoded in the CTX phage [112], and the toxin co-regulated pilus (TCP) [57], while during intracellular infection of eukaryotic cells, *V. cholerae* translocates effectors into the cell to subvert cellular metabolism [9,86].

A key point in the evolvability of *V. cholerae* -as it is the case for many bacteria species-, is horizontal gene transfer (HGT) [14]. The best example of this is likely the fact that the cholera toxin is encoded in a phage [112], but HGT in this bacterium goes well beyond this *passive* form. *V. cholerae* is naturally competent and can internalize DNA from the environment in an active process that is triggered by the presence of chitin [80]. In marine environments, the chitinous exoskeleton of many animals represent an abundant surface where bacterial communities form. Given the common association of vibrios to zooplankton, natural competence is probably expressed frequently in these organisms, playing a central role

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in their lifestyle. The link to this signal makes ecological sense for a free-living marine bacterium: it is only worth paying the cost of expressing the natural competence machinery when living in community, where potentially interesting DNA is close enough to be captured. Strong support to this idea comes from the recent discovery that *V. cholerae* uses a type 6 secretion system as a weapon to kill non-kin surrounding bacteria and steal their DNA [16]. It is clear, hence, that *V. cholerae* has developed sophisticated genetic machineries to exploit HGT extensively as a powerful source of innovative functions.

The variety of ecological niches in which *V. cholerae* thrives, the different genetic armamentarium used for each occasion, and the sophisticated means used to acquire new DNA, are proof of a high degree of genetic plasticity. In an effort to underline the remarkable genetic peculiarities of *V. cholerae*, this review will focus on two genomic features of this bacterium that are especially uncommon in other species: the bipartite architecture of its genome, and the presence of an extremely large genetic memory of adaptive functions: the superintegron (Fig. 1).

The genome of *V. cholerae* is encoded in two chromosomes

The origin of chromosome 2. Bacteria were initially thought to encode their genome in a single molecule of circular DNA. The discovery in 1989 that *Rhodobacter sphaeroides* has its genome split in two unequally sized chromosomes [103] fos-

Fig. 1. Representation of the genomic architecture of *Vibrio cholerae*. Chromosome 1 is larger than chromosome 2. Replication starts at the origin of replication (*ori*) and ends synchronously at the terminus (*ter*). *ori2* is triggered by the replication of crtS, a small DNA segment encoded in Chr1. During fast growth, the multiple firing of *ori1* leads to an increase in the dosage of genes encoded close to the replication origin. Chromosome 2 bears the 120Kb-long superintegron.

tered investigations revealing that multipartite genomes are not uncommon among bacteria. Indeed, approximately 5% of bacterial species harbour more than one chromosome [105,110]. For instance, the *Vibrionaceae* family comprises 9 genera that share a bipartite genomic architecture [35]. The best characterised organism within this family is *V. cholerae*, that has become a model bacterium in the field of study of bacteria with multiple chromosomes. As in the case of *R. sphaeroides*, the two chromosomes of *V. cholerae* are of unequal size, with a 3Mb-long chromosome 1 (Chr1) and a 1Mb-long chromosome 2 (Chr2) [56] (Fig. 1). The origin of this singular genomic architecture seems to be the acquisition and domestication of a large plasmid in the ancestor of all the members of the radiation [56]. Two observations support this theory:

First, the origin of replication of Chr2 (*ori2*) is similar to those of iteron-like plasmids and not of bacterial chromosomes. In these plasmids, a replication initiator protein binds to short direct repeats (iterons) in the origin of replication. Monomeric and dimeric conformations of the initiator protein allow it to act as a promoter or a repressor of replication [62]. Accordingly, Chr2 replication is governed by the initiator protein RctB [36], that is conserved within all *Vibrionaceae*, but shows no homology with other replication initiators [38]. RctB has monomeric and dimeric conformations and controls replication in a concentration dependent manner through its binding to six iterons in *ori2* [62,111]. Control mechanisms for *ori2* are similar to those of iteron plasmids, namely initiator autoregulation, initiator titration and origin handcuffing [33,67,110,111]. This replication system is different to the one of Chr1 (*ori1*), that resembles canonical bacterial chromosome replication origins [36]. *ori1* contains DnaA boxes for the binding of DnaA, the main initiator of replication of bacterial chromosomes, as well as GATC sites for Dam-mediated regulation of replication. *ori1* is similar to *E. coli*'s *oriC* to the point of being exchangeable [65,110]. Accordingly, the Chr1 partitioning protein ParA1, is phylogenetically related to other chromosomal ParAs, while the one encoded in Chr2 -ParA2- branches with plasmid, phage and megaplasmid homologs [56].

Second, there is an asymmetric distribution of the functions encoded in both chromosomes [77]. Chromosome 1 harbours most of the genes thought to be essential for growth and viability as well as genes encoding DNA replication and repair, transcription, translation, cell-wall biosynthesis and a variety of metabolic pathways; while in chromosome 2 there is a higher proportion of uncharacterized genes [56,77]. This observation could be potentially biased by the marked accumulation of genes of unknown function in the superintegron that we will discuss below. Yet, altogether, Chr2 shows a higher plasticity and seems to evolve faster than the more evolutionary stable Chr1, a feature that is also conserved in the case of *Burkholderia spp.* [30,56] and that suggests that secondary chromosomes, like multicopy plasmids, serve as evolutionary test beds for innovation [96].

The conserved genomic architecture among all genera of the *Vibrionaceae* family suggests that the domestication of the meg-
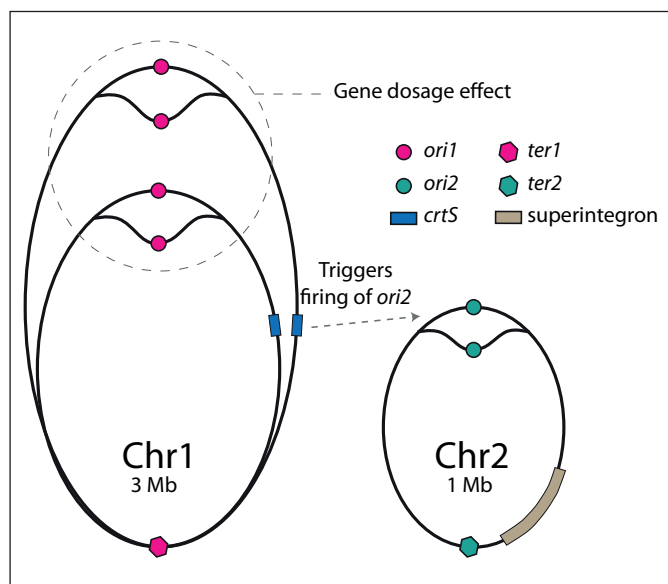
aplasmid that has now become Chr2 likely occurred millions of years ago, before the radiation of the family. The ancient evolutionary link between both chromosomes is also supported by the fact that they both show very similar GC content (46.9% and 47% for Chr1 and 2 respectively) and codon usage bias, contrarily to what is observed when the genome is compared to plasmids eventually found in Vibrios [54]. Such a long common evolutionary history sets a scenario in which stochastic crossovers and genetic rearrangements between chromosome can occur. These events blur some of the rules presented above. Indeed, *V. cholerae* Chr2 does harbour some essential genes, such as the genes encoding ribosomal proteins L35 and L20, and the initiation factor IF3, as well as those encoding the D-serine dehydratase and the threonyl-tRNA synthetase [56]. The stochasticity of such gene movements is supported by the fact that there are 105 potential gene duplications in the *V. cholerae* genome in which copies are located in different chromosomes [56]. Also, the essential genes located in Chr2 in *V. cholerae* are still encoded in Chr1 in other species of the *Vibrio* genus, suggesting that Chr2 was already stable before these genetic rearrangements. Interestingly, there are also examples of regulatory pathways whose genes are split between both chromosomes like the *luxOPQSU* genes involved in the synthesis of the quorum sensing autoinducer molecule AI-2, with *luxOSU* located in Chr1 and *luxPQ* in Chr2 [56].

Altogether, it would seem that the ancestor of the *Vibrionaceae* acquired at some point a megaplasmid that was stable enough to become evolutionary linked to its host. This association was probably strengthened further by the transfer of essential genes from the main chromosome to the plasmid. Yet, since these rearrangements are not conserved among *Vibrio* species, it is unlikely that they were critical for the initial stabilisation of the plasmid, but rather the result of random events occurring between the main chromosome and the already stable megaplasmid.

Management of 2 chromosomes.

In order to efficiently divide, bacterial cells have to replicate their chromosome, physically locate the two copies of each chromosome in precise regions of the cell and trigger the formation of a septum that will isolate the nucleoids and produce the division of two cells with equivalent genetic content. Distributing the genome among two replicons is therefore a complex evolutionary phenomenon, in which replication and segregation of both molecules have to become coordinated and synchronized processes. Indeed, both chromosomes have to be replicated once and only once per cell cycle, and segregated so that the offspring contains one copy of both chromosomes. This choreography occurs in a cell that is extremely smaller than the chromosomes it contains. The orchestration of this phenomenon in *V. cholerae* is complex, yet it is likely the simplest possible model to study the maintenance of multipartite chromosomes. Therefore, any advances in the understanding of *V. cholerae*'s management of its genomic architecture will likely have a profound impact in fields beyond Microbiology, where multipartite genomes are the common rule.

Coordination of replication. Bacterial chromosomes replicate at a very precise moment within the cell cycle, while plasmids seem to replicate independently of it. Having a plasmid origin, Chr2 could potentially replicate independently of Chr1, but it has been shown that this is not the case [37]: co-evolution of the two replicons within the cell has led to a coordination in the replication timing of both molecules. This has provided the first example of communication between chromosomes for replication [87]. Interestingly, rather than starting simultaneously, replication is synchronized to terminate at the same time in both chromosomes [89]. Coherent with the difference in size between both molecules, *ori2* is therefore fired when Chr1 has already replicated 2/3 of its total length. The specific cue triggering Chr2 replication has been cryptic for a long time, and only now we are starting to unveil the molecular basis of this synchronicity. The signal sparking the firing of *ori2* is the replication of *crtS*, a 150bp-long sequence located at 2/3 of the right replichore of Chr1 [108] (Fig. 1). *crtS* is a binding site for RctB, the *ori2* initiator, and it stablishes physical contacts with *ori2* during all the cell cycle [4,108]. The exact mechanism by which *crtS* induces Chr2 replication is not yet understood, but it could involve a structural interplay. Hence, current data suggests that the mechanism is completely novel and its understanding will open new fields in biology.

Segregation choreography. The position and movement of replicating chromosomes along the cell is a well-orchestrated phenomenon governed by a dedicated machinery that assures its correct segregation into daughter cells. These partitioning systems are based on the interaction between ParAB proteins and specific binding sequences, the *parS* loci, encoded in the origin of replication of the chromosome. ParAB are mainly known for their role in replicon segregation, yet they can act as transcriptional repressors to control their own expression levels and that of adjacent genes [90]. Additionally they have an unrelated pleiotropic effect on the transcriptional levels of a variety chromosomal genes [5]. *V. cholerae* contains two sets of ParAB proteins -ParAB1 and ParAB2-, each recognising distinct *parS* sites and segregating its cognate chromosome [115,116]. Chromosomes are longitudinally arranged in the cell, but while Chr1 occupies its hole length, Chr2 seems to be restricted to the youngest half of it (the new pole) [32] (Fig. 2). The *ori1* of Chr1 is anchored to the old pole through the interaction of ParA1 to HubP, a pole anchor protein [114]. After replication, *ori1* starts migrating from the old pole to the new pole. *ori2* follows the same path, but its replication is delayed until 2/3 of Chr1 replication, and its starting point is in the midcell region [32]. While the replication and segregation of both *ori* are separated in space and time, the replication and segregation of the terminus region (Ter) of both chromosomes are synchronized and locate together at midcell [32,89] (Fig. 2). In bacteria with a single chromosome, the Ter region is also located at midcell, and its segregation is synchronized with cell division. Before segregating, spontaneous cointegrates that might have formed through homologous recombination between sister chromatids, have to be resolved by the XerCD proteins. These recombinases release
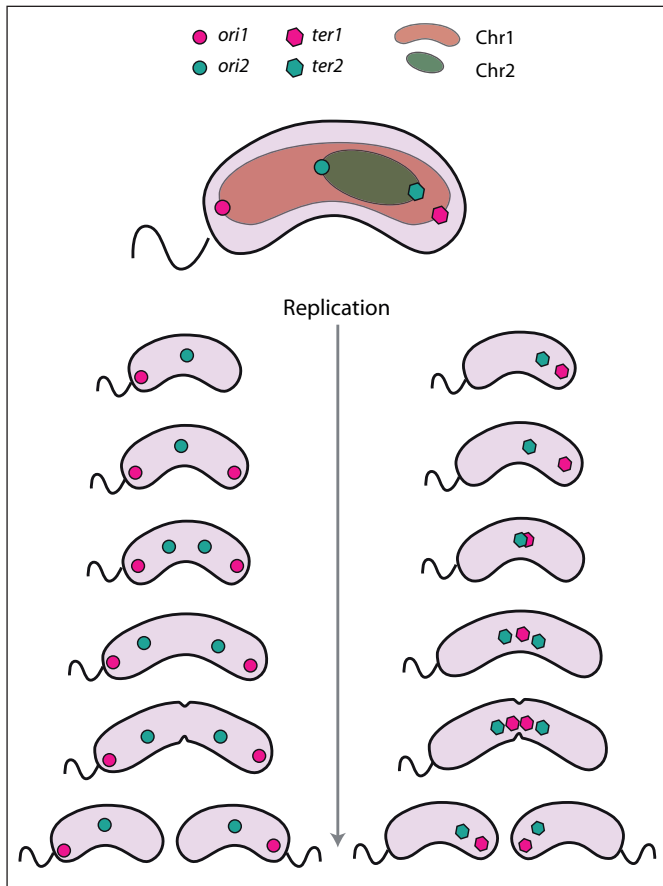
Fig. 2. Spatial distribution of both chromosomes in the *V. cholerae* cell and choreography of *ori1*, *ori2*, *ter1* and *ter2* during chromosome replication and cell division. Modified from [107].

monomeric sister chromatids through a site-specific recombination reaction at the *dif* site located within Ter. *V. cholerae* possesses a single set of XerCD proteins, that act upon both *dif1* and *dif2*. These recombinases are activated by FtsK, a DNA propeller protein that is anchored to the cell membrane where the septum will be formed, and that is capable of searching and locating the *dif* sites by reading specific sequences on DNA [12,13]. This quick translocation of DNA away from where the septum is forming helps pull sister chromatids into their future cellular compartments. Once FtsK arrives at the *dif* site, it triggers XerCD dimer resolution [106], releasing monomeric chromosomes. Cells can then safely divide through the formation of the Z ring promoted by the FtsZ tubulin-like protein.

Advantages of a bipartite genomic architecture.

Given the complexity of the maintenance of two chromosomes, the evolutionary advantage provided by a multipartite genome must be significant in order to drive such a major change. A key element here is that in fast growing organisms, the time needed to replicate the chromosome (taking into account the processivity of DNA polymerases and the size of each replichore of the chromosome) is larger than the doubling

time of the bacterial cell. Bacteria with single chromosomes solve this paradox by firing the origin of replication several times per cell cycle (Fig. 1), so that when a given cell divides, each daughter cell receives a chromosome that has already been partially replicated. The presence of such a mechanism is proof of the adaptive value of fast growth. Hence, it was hypothesized that one of the main advantages of dividing the genome in two replicons could be a shorter replication time and a shorter doubling time. Coherent with this, some Vibrios replicate extremely quickly: *V. cholerae* shows doubling times of 17 minutes [97] -much shorter than the approximately 22 minutes it takes *E. coli* to divide- and *Vibrio natriegens* is the fastest growing organism described to date, with doubling times below 10 minutes [113].

Other traits of the organization of *V. cholerae* genome support the idea that this bacterium is streamlined for fast growth. Bioinformatic studies suggest that in fast growing bacteria, highly used genes tend to locate close to the origin of replication where they can have an increased gene dosage effect during rapid growth [31] (Fig. 1). Indeed, as we saw before, bacteria can fire multiple times the *oriC* during fast growth, in order for chromosomal replication to keep up with cell division. This entails a transient increase in copy number of those genes that are located close to the *oriC*, providing a higher transcriptional activity. This observation was experimentally confirmed in *V. cholerae* [97,98]. The relocation of the S10 locus, that encodes half of the ribosomal proteins, to a variety of distances from the *ori1* produced a distance-dependent increase in doubling time and an attenuation of virulence in an animal model [97]. Hence, gene order in *V. cholerae*'s chromosome is optimised for fast growth and this is key for colonisation. This supports the high adaptive value of such trait and the potential benefit of dividing the genome in two separate replicons.

Overcoming major technical difficulties, the hypothesis that a segmented genome allows *V. cholerae* for a faster growth was actually tested experimentally and ruled out. Val and collaborators produced a *V. cholerae* strain in which both chromosomes were fused together respecting the axial symmetry, gene synteny, strand bias and the polarities of the original replichores [109]. The effect on generation time of such rearrangement was found to be minimal, suggesting that a faster growth is not the force driving the evolution of multipartite genomes and leaving the subject still open for discussion. Coherently with the plasmid acquisition model it is possible that the question about the origin of a multipartite genome should be formulated the other way around: why have the two chromosomes not fused? Indeed, chromosomes can spontaneously form cointegrates through homologous recombination between identical insertion sequences in both replicons. This has been observed in Dam methylase mutants, in which Chr2 replication is compromised [107]. Lethality can therefore be avoided through chromosomal cointegrates that allow Chr2 to be replicated from *ori1*. The viability of chromosome fusions when *ori2* is inactive puts forward that bipartite genomes are probably stable because single chromosomes with more than one active *ori* are unstable.

The superintegron

What is an integron? Chromosome 2 of *V. cholerae* contains a large set of intergenic repeated sequences named *Vibrio cholerae* repeats (VCR), that flank sets of genes that were extremely variable. This structure turned out to be one of the largest integrons known to date, the superintegron [79] and the VCRs were in fact the recombination sites in cassettes. Integrons are memories of adaptive functions that allow bacteria to adapt rapidly to changing environments [20,39,78]. These elements act as genetic platforms for the recruitment and stockpiling of new genes embedded in a small type of mobile genetic element called integron cassettes [50,51]. Integrons are composed of a stable platform and a variable region (Fig. 3). The latter is formed by the array of cassettes that encode a variety of different functions, while the stable platform contains the *intI* gene that encodes the integrase, the insertion site (*attI*) and a dedicated promoter for the expression of cassettes, the Pc [28] (Fig. 3). Indeed, cassettes are normally composed of a promoterless gene and a recombination site (*attC*) so that genes are rendered functional upon integration in the *attI* site, where the Pc promoter ensures their transcription. Recombination in integrons is unique in many ways [19,74], and is the result of an evolutionary innovation process [40]. Cassette recruitment can occur many times, leading to the stockpiling of adaptive functions [26]. As a consequence, a gradient of expression from the Pc is established along the array [28], making the older functions (those acquired first chronologically) to be pushed apart from the Pc by new insertion events, and ultimately become silent [92]. T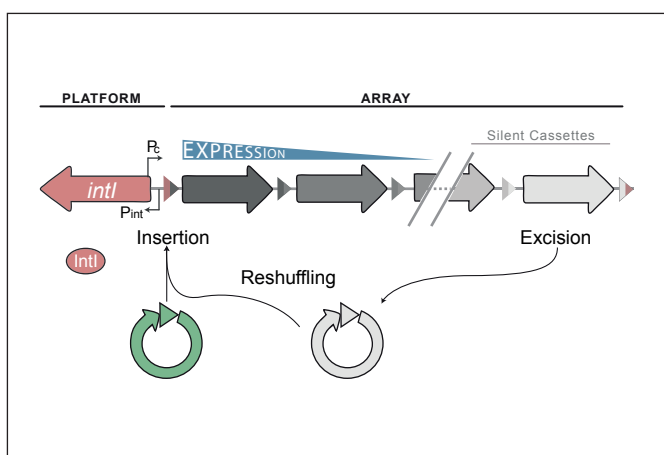hese silent cassettes are therefore carried at the lowest possible cost –the cost of replication- but remain available in case they are necessary. Indeed, under stressful conditions, integrases can randomly excise cassettes from the array and re-insert them in first position, next to the Pc, where their expression is maximal [27]. This reshuffling can help recover a function that was adaptive once, but has been silent for some time [6]. Integrase expression is under the control of the SOS response, a regulatory network that is triggered under stressful conditions and during HGT [6-8], linking the functionality of integrons to the host's needs [48]. The tight control of the integrase seems necessary to keep the fitness cost of integrons low [70]. Indeed, when integrons are found in bacterial species that lack the SOS response -like *Acinetobacter spp.*- integrase genes tend to accumulate disruptive mutations [53,100]. The same happens in the rare cases where the integrase activity is not under the control of the SOS response. This is the case of the class 2 integron integrase (see below), that has been known for many years to contain a premature stop (ochre) codon [52], and that has very recently been shown to escape SOS regulation [63]. The success of integrons is probably the consequence of the adaptive value they provide, their low cost, and the tight intertwining of its activity with bacterial physiology through the SOS response and other cellular mechanisms [39,73,76].

Integrons and resistance. Integrons were first discovered for their role in the rise of multidrug resistance in *Shigella flexneri* isolates during the 1950's in Japan [82,101]. These strains bore the NR1 plasmid, carrying, among other things, a Tn21 transposon that contained what was later classified as a class 1 integron, conferring resistance to aminoglycosides, sulphonamide and biocides [71]. Four more integrons were discovered later and classified as classes 2 to 5, attending to the sequences of the integrase-coding genes [2,29,52,58,99]. They were all related to antimicrobial resistance and were found associated to mobile genetic elements such as transposons and conjugative plasmids, earning them the name of *mobile integrons* (MI). The class 1 MI is the most clinically relevant integron, and has been studied in depth, quickly becoming the experimental model in the field. Further studies revealed the presence of integrons in the chromosomes of many bacterial species, leading to the current understanding that these *sedentary chromosomal integrons* (SCI) are actually the natural form of integrons and the ancestors of MIs [79,91,93]. Indeed, the stochastic mobilization onto plasmids of SCIs allowed them to reach the human environment through food [43,44], where those containing antibiotic resistance cassettes were selected through the high antibiotic pressure exerted by humans in the last decades. The dissemination of integrons is today a major cause of multidrug resistance in clinically relevant strains [69], and currently one of the major threats for modern medicine. The history of the mobilisation of integrons, from the environment through food to the hospital, exemplifies the need for a global view on the ecology of antimicrobial resistance determinants [46] in order to design efficient strategies to fight anti-

Fig. 3. Diagram of the integron. Genes are represented by arrows and recombination sites as triangles. The *intI* gene encodes the integrase that governs cassette insertion and excision. Coupling of both reactions reshuffles cassette order. Cassettes are expressed from the Pc promoter in the integron platform. Multiple insertion events lead to the stockpiling of cassettes, constituting a memory of adaptive functions. Expression of cassettes is weaker when located far from the PC. Integrases are expressed from the Pint when the SOS response is triggered and can reshuffle cassettes, changing their expression levels.

microbial resistance. Such a framework is provided by the "One Health" concept, that postulates the interdependence of human health with the health of animals, food and the environment [21].

Chromosomal integrons: the superintegron.

The first sedentary chromosomal integron described was the superintegron (SI), an extremely long structure found in the secondary chromosome of *V. cholerae* (Fig. 4). It contains 179 cassettes and spans 126 kilobases, comprising 3% of the total DNA of the cell [79]. It is the most variable region of the genome to the point of being useful in the genetic characterization of isolates [24,68]. The superintegron is the best studied chromosomal integron and the paradigm in the field of SCIs. Arguably, this field has not been explored in depth, and clearly lacks experimental evidence for many of the observations performed. This is somewhat surprising if we take into account that integrons have been found in the chromosomes of a variety of major human pathogens, such as *V. cholerae*, *Vibrio vulnificus* [22] or *Pseudomonas aeruginosa* [18]; that they are also closely related to pathogenicity in bacteria affecting crops such as *Xanthomonas*, where cassette content seems to determine the pathovars of the strain [45]; and that they have driven bacterial evolution for eons, and might reveal a myriad of interesting aspects of their biology [78]. Still, most of the studies of SCIs, including the superintegron, are descriptive. Yet they reveal intrinsic and important differences with mobile integrons such as their difference in size, their streamlining to capture cassettes [75], the smaller size of the genes encoded in the SI cassettes, or the difference in the functions encoded. All these observations suggest that many aspects of the biology of these platforms will not be understood if we limit our studies to the class 1 integron.

Cassette functions. Cassette arrays of mobile integrons are almost exclusively devoted to genes conferring antimicrobial resistance. On the other hand, chromosomal integrons contain cassettes of broadly unknown function. 66% of cassettes in SCIs from *Vibrio* species encoded proteins with no homologs in the databases and 12% had homologs of unknown function [18,88]. The paucity of pseudogenes in cassettes, together with structural studies on proteins of unknown function, are proof that these cassettes are functional and subjected to purifying selection. The structure of some proteins encode in cassettes has been solved and show a variety of novel folds [102], suggesting that a myriad new protein families are awaiting to be discovered and characterized. The remaining 22% of cassettes encode genes related with a broad variety of functions such as virulence, DNA modification, toxin-antitoxin systems, phage-related functions, and acetyltransferases [18,88]. With superintegrons containing large arrays of extremely different cassette content from one strain to another, it is clear that the environmental pool of cassettes is a prodigious reservoir of novel protein families and functions of great biotechnological interest [18,95]. Despite our ignorance on the possible functions encoded in cassettes, a highly adaptive ecological value can be assumed. Indeed, while superintegrons from geographically distant isolates of the same species are distinct, highly similar superintegrons can be found among different *Vibrio* species that share the same ecological niche [17]. This suggests a strong selective advantage for cassette bearers and is also proof of an intense local circulation of cassettes. Given the high adaptive value they provide, cassette functions can reveal novel aspects of bacterial physiology, ecology and evolution.

The function of only a handful of the 179 cassettes encoded in the SI has been experimentally elucidated and serve as an example of the broad array of functions that can be encoded in cassettes. The function of several other cassettes found in a variety of other *Vibrio* species has also been elucidated (for a complete list see [88]). Here are the examples of functionally characterized cassettes in *V. cholerae*:

1. Sulphate binding protein (SBP). An SI cassette showing high homology with a SBP from *E. coli* was identified
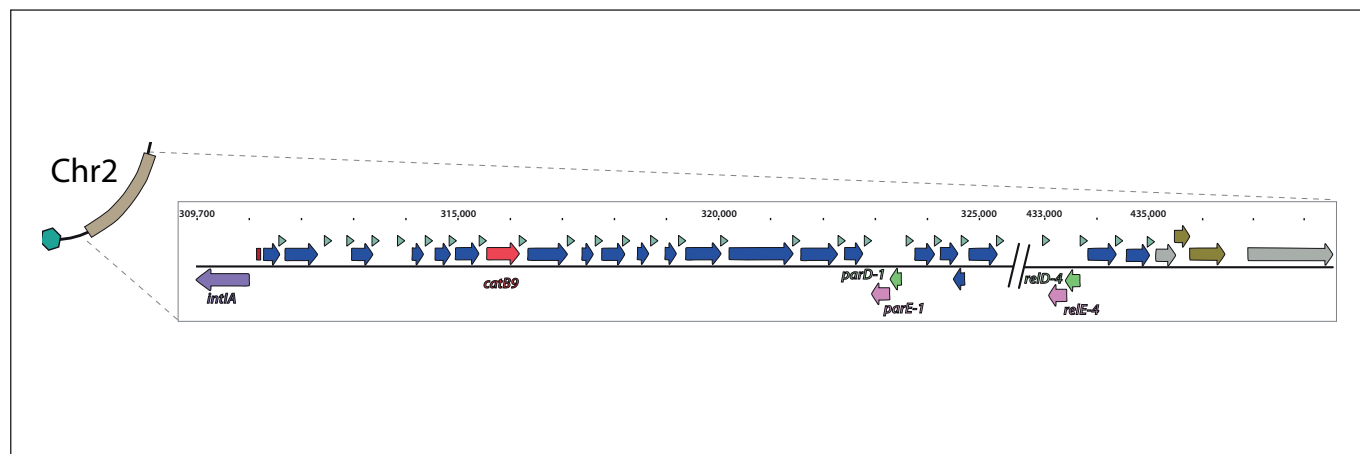

Fig. 4. Scheme of the superintegron. Genes are shown as arrows and VCRs (*attC* sites) as green triangles. Blue: genes of unknown function. Red: chloramphenicol resistance gene. Pink and green: toxin-antitoxins. Yellow: transposase. Grey: genes outside the SI.

in *V. cholerae* [93]. Sulphate uptake is necessary for cysteine biosynthesis and in *E. coli*, it is granted by a sulphate (SBP) and a thyosulphate binding protein (TSBP), encoded respectively in the *sbp* and *cysP* genes. The *sbp⁻*/*cysP⁻* double mutant is auxotrophic for sulphur. The SBP encoded in *V. cholerae* SI restored prototrophy in this mutant when growing on minimal media with sulphate as sole sulphur source, confirming the predicted role of the cassette gene [93].

2. Transcriptional regulator: an approach aiming to solve the crystal structure of cassette-encoded genes identified Cass2, a transcriptional regulator with a fold related to that of AraC/XylS transcriptional activators [34]. The closest homologs of Cass2 are drug-binding regulators. Accordingly, Cass2 is capable of binding cationic drug compounds at nanomolar concentrations. Intriguingly, we ignore the gene or set of genes controlled by this regulator, but the fact that it is encoded in a mobile and accessory region of the genome makes it especially interesting. The structures of two other proteins encoded in *V. cholerae* cassettes have been solved, but their function remains unclear [102]. Instead, this study highlights the prodigious variety of new folds and functions held in integron cassettes.

3. A heat stable enterotoxin similar to that of *E. coli* was identified in non-O1 *V. cholerae* strains. This toxin produces the accumulation of fluids in the intestine of the suckling mouse [3] and is encoded in an integron cassette, as proved by the surrounding VCRs [84].

4. Mannose-fucose resistant hemagglutinin. Hemagglutinins are adhesins that help bacteria to adhere to cells and colonize their hosts. Their activity can be inhibited by certain saccharides, a feature that was used to characterize these proteins. A hemagglutinin that was insensitive to mannose and fucose was first identified in the genome of *V. cholerae* [42] and later linked to VCRs [11]. The gene encoding this protein was later identified as VCA0447 in the chromosome of strain N16961, and the cassette encoding it is located in position 163 within the superintegron. Compared to the WT strain, a mutant of this adhesin showed a drop of 3 orders of magnitude in total cell counts in the intestine of infant mice 20 hours post inoculation [42] proving its major role in the colonization process.

5. *catB9*: The first available genome sequence of a *V. cholerae* isolate, that of O1 El Tor N16961 strain, revealed the presence of three cassettes potentially involved in drug resistance [56]. They encoded proteins showing high levels of homology with chloramphenicol acetyltransferases, fosfomycin resistance proteins and gluthatione transferases. Other acetyltransferases potentially related to antibiotic resistance were also identified [92]. Of all these, only the chloramphenicol acetyltransferase encoded in the *catB9* gene was actually capable of conferring resistance [92].

Interestingly, *V. cholerae* N16961 is susceptible to chloramphenicol because this cassette is located in ninth position of the array, and therefore too far away from the Pc promoter to be transcribed at the levels necessary to provide resistance. Nevertheless, any of the stress signals that trigger integrase activity can produce the rearrangement of cassettes in the SI and lead the appearance of chloramphenicol resistant clones through the relocation of *catB9* closer to the Pc [6]. This cassette can also be captured by mobile integrons borne by conjugative plasmids [92]. This finding provided empirical evidence for the origin of resistance cassettes in mobile integrons. We now understand that mobile integrons circulate among a variety of bacterial species recruiting integron cassettes from their SCIs and bringing them back to the clinical setting, where resistance cassettes provide a selective advantage and are selected for. This is further supported by the presence of *attC* sites in MI cassettes that are virtually identical to those in SCIs [94], as well as by strong differences in GC content and codon usage of genes encoded in MI cassettes, suggestive of cassettes originating in a diversity of genetic backgrounds.

6. Toxin antitoxins: a distinct class of cassettes in *V. cholerae* SI are toxin-antitoxin (TA) systems [49,60]. These modules are known to stabilize plasmids through a post segregational killing mechanism [61]: the antitoxin can counteract the activity of the toxin only as far as it is constantly produced. Indeed, the difference in half lives of the toxin and the antitoxin means that the loss of the TA genes produces a shortage in the supply of the labile antitoxin, that degrades rapidly and allows the more stable toxin to kill the cell. Therefore, any cell that loses a TA system will ultimately die, whether it is encoded in a plasmid or in a superintegron cassette. A variety of 17 TA systems are found distributed along the SI, suggesting that the structure needs strong stabilization systems to be streamlined for genetic capacitance [75] and become such a vast memory of adaptive functions (Fig. 4). Interestingly, since antitoxins need to be expressed constantly, TA systems encode their own promoters. This means that they can modify the transcription activity of genes located downstream, proving that the simplistic model of the integron in which the only promoter is the Pc is likely over-simplistic. Still, many TA modules are actually encoded in opposite orientation compared to the rest of cassettes, and therefore do not promote the expression of any other gene (Fig. 4). This exceptional inverted organization of genes within a cassette, as well as their autonomous transcription, are proof of the peculiar nature of TA systems. This has a profound impact in some fundamental questions of integrons, such as the origin of cassettes. Indeed the fact that TA modules encode their on promoter is one of the main arguments against the RNA-based model of cassette creation, the only hypothesis on the origin of cassettes (for a review see [39]). Also, given the streamlined con-

trol of orientation in the integron [83], it remains cryptic how can a very specific subset of genes show an inverted orientation. TA systems are also relevant because they are known to have functions beyond DNA stabilization. Toxins can be activated through a variety of signals interfering with central metabolic activities in non-lethal ways [23]. TAs are involved in several important processes of bacterial physiology and are also involved in infection and antibiotic resistance. Some examples are the induction of persistence [41,85] (a dormancy state in which the low metabolism of bacteria entails an enhanced resistance to a broad variety of insults), biofilm formation [64], quorum sensing [66], phage resistance [55] and adaptation to physical conditions (temperature, pH...) [72].

Altogether, these six examples are proof of the broad adaptive functions that cassettes can encode. Since the vast majority of cassette functions remain cryptic, research aiming to unveil other functions will certainly produce major advances in our understanding of bacterial evolvability. With almost 200 extremely assorted cassettes per genome, Vibrios possess a virtually infinite reservoir of exchangeable adaptive functions. The superintegron, with its continuous circulation of cassettes among bacteria, and the domestication of a large plasmid to become an intrinsic component of *Vibrio cholerae*'s genome are micro and macro-evolutionary proofs of the exceptionally high degree of genetic plasticity of this bacterium.

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