**Plesiomonas shigelloides**, an Atypical Enterobacterales with a Vibrio-Related Secondary Chromosome

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**Abstract**

About 10% of bacteria have a multichromosome genome with a primary replicon of bacterial origin, called the chromosome, and other replicons of plasmid origin, the chromids. Studies on multichromosome bacteria revealed potential points of coordination between the replication/segregation of chromids and the progression of the cell cycle. For example, replication of the chromid of Vibrionales (called Chr2) is initiated upon duplication of a sequence carried by the primary chromosome (called Chr1), in such a way that replication of both replicons is completed synchronously. Also, Chr2 uses the Chr1 as a scaffold for its partition in the daughter cells. How many of the features detected so far are required for the proper integration of a secondary chromosome in the cell cycle? How many more features remain to be discovered? We hypothesized that critical features for the integration of the replication/segregation of a given chromid within the cell cycle program would be conserved independently of the species in which the chromid has settled. Hence, we searched for a chromid related to that found in Vibrionales outside of this order. We identified one in *Plesiomonas shigelloides*, an aquatic and pathogenic enterobacterium that diverged early within the clade of Enterobacterales. Our results suggest that the chromids present in *P. shigelloides* and Vibrionales derive from a common ancestor. We initiated in silico genomic and proteomic comparative analyses of *P. shigelloides*, Vibrionales, and Enterobacterales that enabled us to establish a list of features likely involved in the maintenance of the chromid within the host cell cycle.

**Key words:** *Plesiomonas shigelloides*, Vibrionales, Enterobacterales, RctB, chromid, SeqA, MatP.

**Significance**

Vibrionales and Enterobacterales are two closely related orders that derive from a common ancestor. Although Vibrionales are multichromosome species, Enterobacterales are known to be monochromosome bacteria. What are the features and factors needed to ensure the sustainability of multiple chromosomes in a cell? We addressed this question by searching and identifying an Enterobacterium with multiple chromosomes, *Plesiomonas shigelloides*, and by carrying out a comparative analysis of its genome and proteome with those of the monochromosome Enterobacterales and the multichromosome Vibrionales.

**Introduction**

Most bacteria are monochromosome bacteria, that is, they carry their core-genome on a unique bacterial chromosome. On the other hand, a fraction of bacteria (called multichromosome bacteria) have a composite genome in which the core-genome is split in two or more replicons. The larger
replicon possesses a replication initiation setting (replication origin and initiator protein) homologous to that found in monochromosome bacteria, whereas the other (usually smaller) replicons are of plasmid origin. They are referred to as chromids. A major criterion distinguishing chromids from plasmids is that the latter are erratically distributed among bacteria whereas chromids are systematically present in all the strains of a given clade, which can be a species, a genus, or an order, like the Vibrionales. Other criteria for qualifying a replicon as a chromid were also proposed such as the transfer of core-genome genes on the chromid, some of which might even be essential (diCenzo and Finan 2017).

Vibrio is an order of gammaproteobacteria that regroups aquatic bacteria, among which the model organism *Vibrio cholerae*, the causative agent of cholera. The genome of Vibrionales is split into a primary circular chromosome (*Chr1*) and a smaller circular chromid, referred to as the secondary chromosome (*Chr2*) (Trucksis et al. 1998; Yamaichi et al. 1999; Kirkup et al. 2010). *Vibrio* are closely related to Enterobacteriales and both orders emerged from a last common ancestor (LCA). In contrast, Enterobacteriales regroups monochromosome bacteria, among which the model organism *Escherichia coli*, suggesting that the *Chr2* of Vibrionales evolved from a plasmid that was either acquired early in the history of the Vibrionales or earlier even, in the LCA and lost secondarily in the Enterobacteriales.

Based on studies of *V. cholerae*, the persistence of *Chr2* in Vibrionales (and prospectively of chromids in general) was associated with the selection of several genetic features ensuring a tight control of its initiation of replication and a finely tuned coordination of its replication/segregation within the host cell cycle (Ramachandran et al. 2016; Espinosa et al. 2017).

In Vibrionales, replication of *Chr2* is initiated once and only once per cell cycle. Replication is triggered at ori2 (an iteron-based origin of replication), whose organization is similar to that found in F and P1 plasmids, and directed by RctB—a homolog of plasmid replication initiator proteins like RepA (Orlova et al. 2017). Replication of *Chr2* occurs during that of *Chr1* and such that the termination of the replication of *Chr1* and *Chr2* are synchronized (Rasmussen et al. 2007). *Chr2* copy number is controlled through plasmid-like molecular mechanisms such as the negative control of replication and the handcuffing of newly synthesized ori2 sisters by RctB dimers (Ramachandran et al. 2016). *Chr2* replication initiation is also controlled by bacterial chromosome-like mechanisms. Some are specified by proteins of the Dam co-factor (Brézellec et al. 2006). The affinity of RctB for the iterons is modulated by the Dam-dependent methylation of ori2, which might be sequestrated by SeqA after replication initiation, like the origin of replication of the bacterial chromosome (Lu et al. 1994). Finally, the perfect timing of *Chr2* replication initiation is dictated by a short sequence located on the main chromosome (crtS), whose duplication triggers replication initiation of *Chr2* (Baek and Chattoraj 2014; Val et al. 2016; Ramachandran et al. 2018). The same coupling seems to operate in all the Vibrionales (Kemter et al. 2018).

*Chr2* segregation is driven by an essential partition machinery, *parAB2*, which uses *Chr1* DNA as a scaffold to distribute newly replicated copies along the cell axis (Yamaichi et al. 2007). Tracking of several loci along *Chr1* and *Chr2* revealed that the terminus region of the two replicons, *ter1* and *ter2*, are synchronously recruited at midcell before duplication, whereas the rest of the genome is already segregated (David et al. 2014). Sister copies of *ter1* and *ter2* are maintained at midcell before cell division by MatP (Demarre et al. 2014), which is part of the dam cohort (Brézellec et al. 2006). In *E. coli*, MatP was shown to exert its function by interacting with specific sequence motifs, the *matS* sites, which are only present in the terminus region of the chromosome (Mercier et al. 2008). The positioning of sister copies of *ter1* and *ter2* at midcell is important for the resolution of RecA-induced chromosome dimers after replication. A site-specific recombination machinery (Xer) acts at specific chromosomal sites, *dif1* and *dif2* located in *ter1* and *ter2*, respectively, through a direct contact with the cell division protein and DNA translocase, FtsK (Val et al. 2008). FtsK is directed toward *dif1* and *dif2* by KOPS sequence motifs located on both arms of the two replicons (Val et al. 2008). The positioning of sister copies of *ter2* at midcell is important for the proper licensing of cell division at the end of the replication/segregation cycle because binding sites for an inhibitor of cell division, SlmA, are found on *Chr2* except for its terminus region (Bernhardt and de Boer 2005; Galli et al. 2016). Thus, a key point of the cell cycle coordination appears to be the synchronized recruitment of the termini of both *Chr1* and *Chr2* at midcell.

Which of the genetic features identified as critical for the maintenance of *Chr2* in *V. cholerae* are common adaptation traits of chromids? We addressed this question by searching for species outside of Vibrionales and carrying a chromid that evolved from the same ancestor plasmid that gave rise to *Chr2* of Vibrionales. We assumed that chromids with common ancestry and orthologous initiator proteins (i.e., initiator proteins with common and identifiable protein domains) would have been integrated within the cell cycle program of the host through the acquisition of similar adaptive modifications that are as many critical features of the replication/segregation of chromids. Hence, we searched RctB-like proteins outside Vibrionales. We identified a RctB-chromid in *Plesiomonas shigelloides*. We established that *P. shigelloides* is an orphan species of Enterobacteriales in which the genome is composed of a main chromosome with a bacterial origin of replication (ori<sup>Chm</sup>) and a chromid whose replication origin (ori<sup>Chd</sup>) is structured like those found on *Chr2* of Vibrionales. The origin of replication of the chromid of *P. shigelloides* is rich in GATC sequences like the origin of the *Chr2* or Vibrionales and its replication terminus region (ter<sup>Chd</sup>) contains numerous MatP-binding sites like the
Plesiomonas shigelloides Contains a RctB-Chromid

The structure of the two central domains (2–3) of RctB is similar with, and common among, plasmid replication initia-
tors (Orlova et al. 2017). Yet, these domains bear a Hidden
Markov Model (HMM) signature distinctive enough in RctB
to be diagnostic of the Vibrionales initiator protein (PF11826 or
DUF3346) (supplementary fig. 1, Supplementary Material online).
We searched and returned from the UniProtKB library other proteins carrying this domain. DUF3346 is present in the
genome of several plasmids that proliferate in Vibrionales and
interestingly in non-Vibrionales species. We returned two posi-
tive hits, one in P. shigelloides and the other in Ferrimonas
marina. Yet, DUF3346 was detected in only one of the six
strains of Ferrimonas available in UniProtKB. As the DUF3346-
containing protein was not identified in all the strains of the
clade, we excluded it from our analysis. In contrast, DUF3346
was identified in each of the four genomes of Plesiomonas
deposited at the time of our analysis in the UniProtKB library.
We confirmed the apparent ubiquitous presence of rctB in
P. shigelloides by PCR amplification in nine strains of the spe-
cies picked randomly in the P. shigelloides collection of Sylvain
Brisse at the Pasteur Institute (Salerno et al. 2007). We further
assessed that it corresponded to the presence of a rctB-repli-
con in each of these isolates by genome sequencing (Materials
and Methods; supplementary fig. 5, Supplementary Material online).

We assembled the genome of strain 7A and used it as a
matrix for the assembly of the ori region of all the other strains
sequenced (Materials and Methods). The presumptive origin
of replication of the RctB replicon corresponded to a large of-
free DNA sequence (~1.6 kb) flanked on one side by rctB and
on the other side by the parAB operon (supplementary fig. 2,
Supplementary Material online). Careful scrutiny revealed a
complex organization of the origin of replication, similar to
that found in the Chr2 of Vibrionales. We identified two types
of conserved iteron-like sequences distributed on each side of a
DnaA box sequence (supplementary fig. 2, Supplementary
Material online). Six 10 mer sequences [5’-TGGATCGT-3’] lay upstream of the rctB-like gene. Strikingly, the 8 mers are regularly scattered, like
the 12 mers in the origin of replication of the Chr2 of the
Vibrionales.

A protein analysis was then carried out on P. shigelloides
RctB. HHpred analysis revealed the presence of two Pfam
domains: HTH_55 and DUF3346, which are diagnostic of
structural domains 1 and 2–3 of RctB of Vibrionales, respec-
tively (supplementary fig. 1, Supplementary Material online).
The analysis predicted also a folding similar to that established
for domains 1 and 2–3 of the RctB of V. cholerae, respectively
(fig. 1a). Finally, we collected the RctB protein sequences of
P. shigelloides, of plasmids and Chr2 of Vibrionales and com-
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protein identified in Plesiomonas forms an evolving branch
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 Altogether, these results indicates that P. shigelloides pos-
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Plesiomonas shigelloides Is an Enterobacterales

Over the last decades, P. shigelloides was alternately ranged in
the Aeromonadales, the Vibrionales and finally in the
Enterobacterales (Janda et al. 2016). The facts that P. shigelloides is found as a free-living organism in aquatic
environments and as an opportunistic pathogen are likely explaining why its classification was challenging.

The presence of a rctB-replicon, otherwise associated with
Vibrionales, led us to reassess phylogenetically the rooting of
P. shigelloides within the gammaproteobacteria. To this end,
we concatenated protein sequences encoded by six house-
keeping genes involved in replication (dnaA, dnaE), transcription
(mfd, rpoB), metabolism (purL), and translation (valS). The
phylogenetic tree obtained shows that P. shigelloides is not included in the main clades of Enterobacterales and Vibrionales.
The phylogeny of selected proteins of the DNA maintenance
program, and in particular those important for the replication/segregation of Chr.2 in V. cholerae, like those of the dam
cohort (Brézellec et al. 2006), supports this conclusion (sup-
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branch of Vibrionales from the LCA of the whole clade.
Several markers support this conclusion. The acquisition of
secM and the absence of a parAB operon on the main chro-
mosome of P. shigelloides is characteristic of the genomes of
Enterobacterales. The absence of a genomic Ter/tus system
(Galli et al. 2019), the persistence of the γ-Flagellar system
(Ferreira et al. 2021), and the arrangement of the ori region of
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speciation of P. shigelloides within the branch of
Enterobacterales (inset in fig. 2). Altogether these results
suggest that the most parsimonious hypothesis is that
P. shigelloides is not a Vibrio but an Enterobacteriales.

We aimed to delineate phylogenetically the group of
Plesiomonas-related species carrying a chromid to investigate
the history of this branch of Enterobacteriales. Yet, the closest
species identified were all well-known monochromosme-
Enterobacteriales. We investigated the possibility that the
genus Plesiomonas regrouped various species and concluded
that the branch of P. shigelloides carries only a single species
(supplementary material and fig. 4, Supplementary Material
online). Hence, P. shigelloides is an Enterobacteriales that
carryings its genome a chromid whose replication initiation
machinery is related to that of the Vibrioales. This situation
is therefore particularly suitable to pursue our comparative study
on the adaptive features needed to ensure the persistence of
a chromid without interfering with the progression of the host
cell cycle in Vibrio and in Plesiomonas.

The Plesiomonas and Vibrio Chromids Carry Common
Replication Regulation Features

The presence of GATC sites in the 8 and the 10 mers iteron-
like sequences within ori_Chm of P. shigelloides is strikingly
reminiscent of the organization of ori2 of V. cholerae
(fig. 3a). It suggests that the affinity of RctB for the iterons
at ori_Chm might be modulated by the methylation status of
the origin of replication, in a way similar to that described in
V. cholerae. It suggests that dam-dependent activities might
be involved in the initiation control of the P. shigelloides
chromid replication.

Another feature of the replication program in Vibrioales is
that chromid replication occurs during that of Chr1. The co-
ordination is such that the termination of the replication of
the two chromosomes are synchronized, suggesting that it
might have been an adaptation of the replication program of
the chromid to the bacterium cell cycle. We performed a
marker frequency analysis in P. shigelloides to investigate
the timing of the replication of the chromid with respect to
that of the main chromosome. We analyzed the nine strains
of P. shigelloides described earlier (Materials and Methods).
The DNA analyzed was extracted from exponentially growing
cells and subjected to deep sequencing (Materials and
Methods). Then, we used the genome of strain 7A as a matrix
for the MF analysis of the eight other strains (Materials and
Methods). Except in the strain NRC32124, the replication of
the chromid occurred during that of the main chromosome,
indicating that the completion of the replication of the main
chromosome is not synchronized with that of the chromid in
all the strains analyzed (fig. 3d; supplementary fig. 5,
Supplementary Material online). The MF profile from
NRC32124 cells is different from those obtained with the
other strains, suggesting that the replication of the chromid
occurred after completion of the replication of the main chro-
mosome. Alternatively, it could indicate that the chromid was
either lost or not replicated in a fraction of the cultivated cells.
Yet, we could not purify chromid-less cells, suggesting that
chromid-less cells are unviable.

The Plesiomonas and Vibrio Chromids Carry Common
Segregation Features

We searched the genome of P. shigelloides for other markers
that might be important for the adaptation of the chromid to
the cell cycle. Chromosome dimers occur during replication in
a RecA-dependent manner. We searched the presence of dif
sites in the replication fork-convergence region of the two
replicons (fig. 3b). We identified a dif site on each replicon: A
canonical site (dif_Chm) was found in the terminus region of the
main chromosome and a more degenerated site (dif_Chm) was
identified in the terminus region of the chromid (fig. 3b). In
support, we identified numerous polarized KOPS on the bac-
terial chromosome and on the chromid pointing toward dif_Chm
and dif_Chm, suggesting that chromosome dimer resolution on
both replicons is FtsK/XerCD-dependent (fig. 3b). Also, we identified numerous SlmA-binding sites (SBS) around oriChm suggesting an active nucleoid occlusion program in P. shigelloides. The situation was much less clear with respect to the presence of SBS on the chromid, likely as a consequence of the small size of the chromid (fig. 3c). Finally, we found that the terminus region of both chromosomes of P. shigelloides are both enriched in MatP-binding sites (fig. 3c). Altogether, these elements strongly suggest a long-standing relationship between the RctB-replicon and P. shigelloides and an advanced adaptation of the chromid to the cell cycle of P. shigelloides.

DNA Maintenance Proteins of Vibrio and Plesiomonas Share Common Protein Signatures

Due to the integration of a chromid in the cell cycle of P. shigelloides, some DNA maintenance genes might have become essential, others might have acquired adaptive mutations.

We addressed the first question by determining the genes that could, or could not, be interrupted by Transposons in a Tn-seq experiment (Materials and Methods). We did not detect any Tn insertion in rctB and in the chromid parAB genes, indicating that the chromid is essential for the viability of Plesiomonas (fig. 4). seqA and dam were shown to be essential in V. cholerae but not in the monochromosomal bacteria E. coli. It is supposedly due to the implication of the gene products in the replication of Chr2. In contrast, Tn-seq data indicated that seqA and dam are facultative in P. shigelloides. Yet, the Tn integration frequency in dam was significantly lower than that expected for a nonessential gene, suggesting that the fitness of a dam null-mutant was reduced in P. shigelloides (fig. 4).

We initiated a comparative analysis of the sequence of various proteins of the DNA maintenance program of P. shigelloides, Vibroniales, and Enterobacterales. In particular, we searched P. shigelloides proteins for motifs that are typically present in Vibroniales and not in Enterobacterales proteins. The presence of matS sites in terChm, the numerous

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**Fig. 2** — Plesiomonas is an atypical Enterobacterales. Phylogenetic tree based on a concatenation of DnaA, DnaB, DnaE, and DnaX protein sequences. Monophyletic clades corresponding to known orders of gammaproteobacteria are indicated: Enterobacterales (ENT), Vibroniales (VIB), Aeromonadales (AER), and Alteromonadales (ALT). Pseudomonas aeruginosa PAO1 was used as an outgroup. Species whose biotope is aquatic are in blue and those in which a RctB-replicon was detected are highlighted in blue. Relevant bootstrap scores and a scale indicative of the substitution frequency are provided. Some genomic modifications that occurred through evolution are reported in the inset. The acquisitions and losses of genes are in green and red, respectively. XoriC correspond to the recombination that occurred at oriC (supplementary fig. 2, Supplementary Material online). β-flg and γ-flg refer to the peritrichous and polar flagellar system, respectively (Ferreira et al. 2021). Yersinia pestis, Proteus mirabilis H4320, Pragia fontum, Plesiomonas shigelloides 7A, Photobacterium laumondii subsp. laumondii TT01, Escherichia coli K12, Erwinia billingiae Eb661, Dickeya dadantii 3937, Citrobacter rodentium ICC168, Cedecea neteri, Vibrio vulnificus YJ016, Vibrio tasmaniensis LGP32, Vibrio nigrirpelchritudo, Vibrio mangrovi, Vibrio cholerae serotype O1 El Tor Inaba N16961, Photobacterium profundum S99, Gramontia hollisae CIP 101886, Alivibrio fisheri ES114, Shewanella oneidensis MR-1, Psychromonas ingrahamii 37, Pseudoalteromonas haloplanktis TAC 125, Idiomarina loihiensis L2-TR, Ferrimonas marina, Colwellia psychrerythraea 34H, Agarivorans albus MKT 106, Tolumonas auensis TA4, Oceanimonas sp. GK1, Aeromonas hydrophila subsp. hydrophila NCIMB 9240, Pseudomonas aeruginosa PAO1.
FIG. 3—Organizational features of the genome of *P. shigelloides*. (a) Organization of the presumptive origin of replication of the replicon of *P. shigelloides*. The presumptive origin of replication of the replicon of *P. shigelloides*, spanning the noncoding sequence from *rctB* to *parAB* is displayed with salient features: GATC sites (*), iterons (colored arrowheads; the sequence of the two types of iterons is provided), DnaA box (green square). The A/T rich region is shown. (b) Calculation of the GC skew and cumulative GC skew on the main chromosome (3,555 kb) and the chromid (299 kb) and distribution of the KOPS on each replichore of each replicon. Each vertical bar represents an occurrence detected on the (+) strand (blue) and the (−) strand (red). Origin of replication on the bacterial chromosome (*oriChm*) and upstream of the *rctB* gene on the chromid (*oriChd*) are identified. The *dif* sites located on the bacterial chromosome (*difChm*) and on the replicon (*difChd*) are also identified. The sequences of *difChm* and *difChd* are provided. (c) Distribution of the SBS and *matS* on the two replicons. (d) Marker frequency analysis of *P. shigelloides* replication program. The analysis performed on DNA of exponentially growing cells follows the replication of the main chromosome (Chm) and of the RctB-chromid (Chd).

FIG. 4—The chromid of *P. shigelloides* is essential for cell viability. Comparison of transposon insertion profiles of *P. shigelloides*, *V. cholerae*, and *E. coli* at *rctB*, *parAB*, *seqA* and *dam* genes (supplementary table 4, Supplementary Material online). The amplitude of the signal represents the relative insertions rate of Transposon at a given position (insertion at a given site/total number of reads). Data from *P. shigelloides* and *V. cholerae* correspond to the combination of three independent experiments. Those of *E. coli* correspond to a single Tn-seq experiment. Blue and pink bars indicate insertions in the forward and reverse strand, respectively.
GATC sites within oriChd together with the reduced fitness of the dam mutant led us to focus on the genes of the dam-cohort (Brézellec et al. 2006).

We detected a Vibrionales pattern in two proteins of P. shigelloides: PsSeqA and PsMatP. The alignment of SeqA proteins of Enterobacterales and Vibrionales revealed the strict conservation of a stretch of 11 amino acid long in Vibrionales—but not in Enterobacterales proteins, within the linker (connecting the NTD and the CTD of the protein) that was shown to be critical for the formation of oligomers of SeqA (Chung et al. 2009). Strikingly, this pattern was also found in PsSeqA, although the protein is clearly phylogenetically rooted to the Enterobacterales clade (fig. 5). Similarly, PsMatP carries a protein motif diagnostic of the MatP proteins of Vibrionales, which is located within the MatP dimerization domain (fig. 6)(Dupaigne et al. 2012).

**Order-Specific Domains Enriched in Ecological Niche-Relevant Activities**

The presence of a rctB-chromid in P. shigelloides led us to consider that the LCA of Enterobacterales and Vibrionales might have already carried a rctB-chromid. According to this scenario, the nascent group of Enterobacterales split early in two sub-branches. One sub-branch lost secondarily the rctB-chromid to give the clade regrouping bona fide Enterobacterales, whereas the other sub-branch gave P. shigelloides. Hence, we hypothesized that P. shigelloides might have retained through evolution activities that are important for the maintenance of the chromid and that might have been lost by lack of pressure of selection in the bona fide Enterobacterales, that is, in Enterobacterales that lost the chromid. To investigate this hypothesis and identify these activities, we developed a global approach based on the analysis of the distribution of Pfam domains in these species.

Pfam domains are autonomous structural and/or functional elements of proteins defined by their HMM signature. Conveniently, their signature persists through evolution almost independently of their degree of relatedness. Proteins may contain several domains and each domain may be present in several proteins, making the distribution of domains within a proteome a handy marker of its history. We defined the “domainome” of an individual as the set of domains present in its proteome and by analogy with genomic cladistics, we defined “pan-domainome” and “core-domainome” as the set of domains found in the proteome of at least one and of all individuals in a given clade, respectively. Because domains are acquired and/or lost through vertical and/or horizontal transfer, the core-domainome regroups the domains that are expected to be under pressure of selection in the species composing the clade. Conversely, the domains of the core-domainome of a given clade that are systematically absent from the pan-domainome of a different but closely related group of species (i.e., with a common ancestor) are likely to be clade specific features.
We established the core- and the pan-domainomes of the Enterobacterales and the Vibrionales out of 21 proteomes of each order (fig. 7; supplementary table 1, Supplementary Material online). The choice of these proteomes was based on the representativity of the species picked and on the completeness of their genome assembly (supplementary table 1, Supplementary Material online). The pan-domainome of the Vibrionales (VIBpan) and Enterobacterales (ENTpan) regroups 4,422 and 4,515 domains, respectively, whereas the core-domainome of the same orders contains 1,282 (ENTcore) and 1,389 (VIBcore) domains. In total, we identified 12 and 22 order-restricted domains within the core-domainome of the Enterobacterales (E-rtd) and the Vibrionales (V-rtd), respectively (fig. 7a).

The distribution of the V-rtd domains contrasts with that of the E-rtd domains. Most E-rtd (8 out of 12) are strictly restricted to this order. None of them was found in P. shigelloides, suggesting that these domains were acquired recently (fig. 7b). In comparison, and except for the two domains present in RctB (HTH_55 and DUF3346; supplementary table 1, Supplementary Material online), all V-rtd domains are broadly distributed among bacteria, archaeae and eukaryotes and 14 out of 22 were identified in P. shigelloides (fig. 7b; supplementary table 1, Supplementary Material online).

Altogether, these results reveal two distinct trends. V-rtd domains were likely present in the LCA, maintained under pressure of selection in Vibrionales and lost by waves in the branch leading to the Enterobacterales: Eight were lost early after the isolation of the Enterobacterales branch along with the main chromosome parAB operon (fig. 7b, inset in fig. 2) and 14 were lost after the separation from the P. shigelloides branch (fig. 7b). In contrast, the majority of the Enterobacterales-restricted domains (8 out of 12) were acquired recently in the Enterobacterales, after the separation from the P. shigelloides branch (fig. 7b).

Discussion

In this report, we established that a chromid related to the Chr2 of Vibrionales belongs to the genome of P. shigelloides. We demonstrated that P. shigelloides is an atypical Enterobacterales through phylogenetic analyses based on various molecular markers, genomic reorganization scars and gene loss or acquisition. This led us to initiate a comparative analysis to evaluate the relevance and the ubiquity of the various genetic traits that were proposed to contribute to the maintenance of chromids in Vibrionales.

Several genomic structural elements typically identified on the main chromosome were also detected on the chromid,
suggesting that they are a requirement for their maintenance. Numerous KOPS were found on each replichore and the presence of a *dif* sequence strongly suggests that chromid-dimers in *P. shigelloides*, like in *V. cholerae*, are resolved through the XerCD recombinase system. Also, the large number of matS sites within the replication fork convergence region suggests that the newly replicated copies of the terminus region of the *P. shigelloides* chromid are positioned at midcell through the matS/MatP system. We found, however, only two SBS motifs on the chromid, suggesting that it is unlikely to contribute to the proper licensing of cell division by nucleoid occlusion. We showed that the termination of the replication of the chromid is not synchronized with that of the bacterial chromosome and furthermore that the timing of replication initiation varied from strain to strain. Nevertheless, the copy number of the RctB-chromid was modeled on that of the main chromosome (supplementary fig. 5, Supplementary Material online). It is too early, however, to conclude that synchronization of the termination of replications is not an important feature of chromids. As mentioned earlier, we observed a deficit of RctB-chromid replication in cultivated NRC31124 cells. We identified a strain of *Plesiomonas shigelloides* without RctB-chromid (NCTC10360). We resequenced this strain to confirm that no part of the chromid was integrated on the main chromosome. We observed that the growth of these two strains (NRC31124 and NCTC10360) was extremely poor (data not shown) and no chromid-less NRC31124 cells could be purified. This indicates that chromid loss seriously and durably compromises the fitness of *P. shigelloides*. Alternatively, it may reflect the fact that the replication of the chromid is still not completely integrated within the host cell cycle.

We identified two genetic signatures that might reveal cellular adaptations to the integration of the replication/segregation of the chromid in the host cell cycle. The first was identified in SeqA. We noticed a protein motif in the linker of *Ps*SeqA similar to that found in Vibrionales. The function of the linker was associated with the flexibility of SeqA in *E. coli*. Active SeqA exist as dimers, in which the NTDs ensures the interaction between two subunits whereas the CTDs interacts with two distinct hemimethylated GATC sites. EMSA experiments revealed that the linker enables long distance interactions between dimers of SeqA and two hemimethylated GATC sites (Guarné et al. 2005). In Enterobacterales, the linker is unstructured and its sequence is random, consistent with its role in the plasticity of the protein. In contrast, the linker found in the SeqA proteins of Vibrionales and *P. shigelloides*, contains a highly conserved protein motif, indicating that a strong pressure of selection is applied on this sequence in this group of species. It would be interesting to investigate whether the linker of SeqA in Vibrionales and in *P. shigelloides* specifies an additional function in connection with the maintenance of the RctB-chromid. The second genetic signature typical of the Vibrionales that was found in a *P. shigelloides* protein is located within ZapB-binding domain of MatP, at the Carboxy terminal end of helix α5 (Dupaigne et al. 2012). Indeed, *PsMatP* falls cladistically within the group

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**Fig. 7**—*Plesiomonas shigelloides* possesses Vibrionales- and Enterobacterales-restricted domains. (a) The core/pan-domainome of the Enterobacterales and Vibrionales were built to identify the Enterobacterales- and the Vibrionales-restricted domains. Thirteen Enterobacterales (E-rtd) and 22 Vibrionales domains (V-rtd) were identified as “restricted” to each order, respectively. Among these domains, some were found in *P. shigelloides* (E-rtd⁺ and V-rtd⁺) and some were not (E-rtd⁻ and V-rtd⁻). (b) Plausible history of the gains and losses of E-rtd and V-rtd domains through evolution since the LCA. Stroke thickness is an arithmetic function of the number of domains.
of the Vibrionales. In *E. coli* and on Chr1 of *V. cholerae*, the segregation of the Ter macrodomain is delayed through a MatP-dependent interaction with the divisome at midcell. Although MatP appears to be also involved in the positioning of the chromid of *V. cholerae*, the connection between the two newly replicated chromid Ter regions was shown to be looser, suggesting an adaptation of MatP to the segregation of two types of replicon (Demarre et al. 2014). Given the constraint similarities in term of genome maintenance between *V. cholerae* and *P. shigelloides*, it would be interesting to investigate whether the common genetic signature detected within the dimerization domain of MatP could be an adaptation to the segregation of the two replicons.

The domainome approach aimed at identifying relevant activities with respect to the maintenance of RctB-chromid led to the identification of two domains of unknown function: PF12069 and PF12119. The proteins associated with these two domains are present in the proteome of all Vibrionales investigated and in *P. shigelloides* and define a monophyletic group (supplementary fig. 6a and b, Supplementary Material online). The PF12119-containing gene is especially interesting because it is distributed typically like a mobile element outside the Vibrionales/Plesiomonas group, suggesting that the gene is under pressure of selection and inherited vertically in the Vibrionales/Plesiomonas group. It could be linked to an implication on the maintenance of the RctB-chromid. Alternatively, the acquisition of these genes could result from the adaptability of the bacterial genomes to their environment. Indeed, six Vibrionales-restricted domains found in *P. shigelloides* are protein components of the polar flagellum. The H- and T-ring contribute to the formation of a periplasmic basal disk associated with flagellar motor, whereas MotY, FlgO, and FlgT assist the assembly of the flagellum (Beeby et al. 2016). Polar flagellum systems are found in several orders of the marine *γ*-proteobacteria (Vibrionales, Aeromonadales, Alteromonadales, or Pseudomonadales). This type of flagellum develops a motor of higher torque than that found in Alteromonadales, or Pseudomonadales). This type of flagellar motor is restricted domains found in *P. shigelloides* and *P. shigelloides*. The acquisition of these genes could result from the adaptability of the bacterial genomes to their environment. Indeed, six Vibrionales-restricted domains found in *P. shigelloides* are protein components of the polar flagellum. The H- and T-ring contribute to the formation of a periplasmic basal disk associated with flagellar motor, whereas MotY, FlgO, and FlgT assist the assembly of the flagellum (Beeby et al. 2016). Polar flagellum systems are found in several orders of the marine *γ*-proteobacteria (Vibrionales, Aeromonadales, Alteromonadales, or Pseudomonadales). This type of flagellum develops a motor of higher torque than that found in *E. coli* and *Salmonella typhimurium*, which appears to be particularly adapted for fast movements in an aquatic environment. Enterobacteriales—like *E. coli*—on the other hand lost the *γ*-proteobacterial polar motor during evolution, probably while adapting to their enteric niche. They acquired through horizontal transfer a more suitable *β*-proteobacterial flagellar system (Ferreira et al. 2021).

**Concluding Remarks**

It is difficult to perceive the immediate interest for the cell to ensure the maintenance of an additional essential replicon, especially if its proper maintenance requires the adaptation of replication-related genes. Several hypotheses were proposed to justify the advantage for a cell to have a multipartite genome like extending the size of the genome, or accelerating the growth rate or even helping the cell adapt to new biological niches. It was also suggested that chromids might be an interesting mean to modulate protein expression by gene dosage. Yet, several counter examples challenge each of these hypotheses (d’Cenzo and Finan 2017), not mentioning that the high percentage of bacterial species, whose genome is contained on a single chromosome (~90%) rather suggests that multipartite genomes are accidental situations, imposed and enforced by the plasmid through toxin/antitoxin genes, for example. The strategies aimed at integrating the replication of a chromid within the host cell cycle might simply seek to perturb it as minimally as possible.

**Materials and Methods**

**Strains, Media, and Growth**

Nine strains of *Plesiomonas shigelloides* were analyzed in this study (the place of sampling is indicated under bracket). NRC32124 (river, Czech republic), O164 (fish, Sweden), NRC2599 (lake, Slovakia), 8A (lake, Sweden), 7A (river, Sweden), NRC73297 (river, Slovakia), NRC89372 (sewage, Slovakia), NRC88238 (lake, Slovakia), and R529480 (human, Slovakia). Strain of *V. cholerae* used for the Tn-seq. For Tn-seq analysis *E. coli* J2163 + pEE18 was used as donor strain. The strains of *P. shigelloides* used in this study were grown in LB at 25 °C. When needed, media was supplemented with Chloramphenicol (25 μg/ml), Kanamycin (50 μg/ml), and t-amino-pimelic acid (0.3 mM). Solid media contained 1.5% agarose.

**Domainome**

**Data Sets**

Genomes of Enterobacteriales used in this study: *Citrobacter koseri* (strain ATCC BAA-895/CDC 4225-83/SG56496), *Cedecea daviae* DSM 4568, *Klebsiella pneumoniae* subsp. *pneumoniae* (strain ATCC 700721/MGH 78578), *E. coli* (strain K12), *Erwinia tasmaniensis* (strain DSM 17950/CIP 109463/ET1/99), *Tatumella ptyseos* ATCC 33301, *Pantoea ananatis* (strain LMG 20103), *Pectobacterium atrosepticum* (strain SCRI 1043/ATCC BAA-672), *Erwinia carotovora* subsp. *atroseptica*, *Brenneria goodwinii*, *Dickeya dianthii* (strain 3937), *Erwinia chrysanthemi* (strain 3937), *Lonsdalea quercina*, *Yersinia pestis*, *Serratia fonticola* AU-P3(3), *Rahnella aquatilis* (strain ATCC 33071/DSM 4594/JCM 1683/NCNR 10570/NCIMB 13365/CIP 78,65), *Hafnia alvei* FB1, *Edwardsiella ictaluri* (strain 93-146), *Morganella morganii* subsp. *morganii* KT, *Photobacterium luminescens* subsp. *laevis* (strain DSM 15139/CIP 105565/TT01), *Xenorhabdus bovenii* (strain SS-2004), *Pragia fontium*, *Leminorella grimmii* ATCC 33999 = DSM 5078, *Plesiomonas shigelloides* 302-73, *Plesiomonas shigelloides* 7a. Genomes of Vibrionales used in this study: *Alivibrio fischeri* (strain ATCC 700601/ES114), *Vibrio*
fischeri, Grimontia hollisae CIP 101886, Photobacterium damselae subsp. damselae CIP 102761, Photobacterium profundum (strain SS9), Enterovibrio nigricans DSM 22720, Vibrio aerogenes CECT 7868, V. cholerae serotype O1 (strain ATCC 39315/El Tor Inaba N16961), Vibrio azureus NBRC 104587, Vibrio fumissii (strain DSM 14383/NCTC 11218/VL 6966), Vibrio nigripulchritudo, Vibrio parahaemolyticus serotype O3: K6 (strain RIMD 2210633), Vibrio diazotrophicus ATCC BAA-2122, Vibrio splendidus YJ016, Vibrio xiamenensis, Vibrio variabilis, Vibrio caribbeanicus ATCC BAA-2122, Vibrio metchnikovii CIP 69.14, Vibrio diazotrophicus, Vibrio nereis, and Vibrio palustris.

Pfam Annotations
The Pfam annotations pertaining to the above-mentioned bacteria were downloaded from the Pfam site (http://pfam.xfam.org/) release 34.0.

Computation of “Order-Specific Domains”
Order-specific domains were obtained using “DomainSiev,” a software developed in 2006 (Brézélec et al. 2006). “DomainSiev” works with Pfam protein domains. It collects protein domains that are systematically present in a set of “in-group” organisms and excludes those that are also identified in at least one organism of the “out-group” set of organisms. Thus, for instance, the computation of E-rtd domains is obtained using the intersection of the set of Enterobacterales organisms as “in-group” and the union of the set of Vibrionales organisms as “out-group.”

Phylogenetic Analyses
An alignment of proteins (a crude alignment generated using the program ClustalW and refined by hand) was fueled into PhyLM (v.3.0) and 100 bootstrap replicates were generated for each analysis (Guindon and Gascuel 2003). A consensus tree was eventually obtained by running the program CONSENSUS and fed as an input tree into PhyML. Significant bootstrap scores (arbitrarily above 70%) are indicated.

Marker Frequency Analysis
Overnight cultures of P. shigelloides were diluted in 10 ml of fresh media to a final OD600 0.01. Cells were grown until they reached an OD600 0.1 and rediluted in 10 ml of fresh media to a final OD600 0.01. Once cultures reached OD600 0.1, cells were collected and subjected to genomic DNA extraction. Extraction of gDNA was performed using the Sigma GenElute bacterial genomic DNA kit and sequencing libraries were prepared using the NEBNext Ultra II FS DNA Library Prep Kit for Illumina kit. Paired-end sequencing was performed in a NextSeq (Illumina). Number of reads used for the MFA of each strain analyzed: PSH1 (34629688), PSH2 (31705987), PSH3 (27088120), PSH4 (36719732), PSH5 (42314957), PSH6 (28884789), PSH7 (31947357), PSH8 (29756969), and PSH9 (33285060). Sequence data analysis was performed as described in Galli et al. (2019) with small modifications.

Reads of all P. shigelloides strains were aligned on P. shigelloides PSH5 genome and sequences not aligned were filtered out. Enriched regions were calculated using a 2- and 500-kb window, values from 2-kb window deviating more than 16% from 500-kb window were discarded.

Tn-Seq Analysis
Samples for Tn-seq analysis were prepared as described (Espinoza et al. 2020). Libraries were paired-end sequenced in a Next-Seq (Illumina). Sequencing data analysis was performed as described (Espinoza et al. 2020). Transposon insertions at dam, seqA, and rctB genes were visualized using ARTEMIS (Carver et al. 2012).

Sequencing
Genomic DNA extracted from E. coli was used for Illumina or Oxford nanopore Technologies (ONT) sequencing library preparation. Illumina libraries were prepared following the standard TruSeq protocol with mechanical shearing (Covaris S220), end-repair, A-tailing and adapter ligation followed by PCR amplification. Illumina libraries were sequenced on a NextSeq500 platform (paired end 2 × 75 bp). For nanopore sequencing library preparation the SQK-NSK007 kit from ONT was used with the corresponding protocol. DNA was sheared using Covaris g-TUBEs to generate approximately 8 kb fragments followed by the optional DNA repair step, end-repair, and adapter ligation. The resulting library was loaded onto a MiniION flow cell version 7.3 and sequenced on a MiniION Mk18 sequencer. The ONT run generated 28 194 “2D” reads (~137 Mb). Base calling was performed using the Oxford Nanopore base callers (Metrichor). Canu was used for correction, trimming, and assembly of reads to produce one assembly of three contigs (3,555,866, 298,933, and 5,987 pb) (Koren et al. 2017). Pilon was used for assembly polishing with the Illumina sequences described above. We performed six rounds with “bases” mode correction and one final round with “all” mode correction (Walker et al. 2014).

Plesiomonas shigelloides 7A Reannotation
The main annotations of P. shigelloides 7A was performed on the MicroScope annotation platform. For proteins annotated as “unknown function” by Microscope, we triggered a reannotation pipeline in order to attribute, when available, Pfam domains to these proteins. To achieve this goal, the HHsuite package was used as follows:

1. Using HHBlits, the primary sequence of each protein annotated as “unknown” was ran against the UniRef30
database in order to create a multiple sequence alignment (MSA).
2. An HMM profile was then then created from this MSA.
3. To improve the robustness of the profile, the secondary structure prediction of the unknown protein was integrated to the HMM.
4. The profile obtained was compared with the Pfam database (32.0) with HHSearch.
5. We wrote a python script to collect the protein sequences in which we identified Pfam domains whose probability to be present in the given protein was above 99.00%. When more than one such domain were identified in the same protein sequence, the domain with the highest score was retained.

**Supplementary Material**

Supplementary data are available at Genome Biology and Evolution online.

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**Data Availability**

The sequence of the strain *Plesiomonas shigelloides* 7A was deposited in the NCBI genome databank (https://www.ncbi.nlm.nih.gov/assembly/GCF_020991025.1). The strains and crude sequences of the *Plesiomonas shigelloides* other than the strain A7 are available upon request to the corresponding author.

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