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REVIEW PAPERS

Additional Chromosomes in Bacteria: Properties and Origin

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Abstract—The review considers papers published over the last 15 years that deal with the presence in cells of some bacterial genera and species of a second chromosome that is smaller than the main one (occasionally, of two additional chromosomes). These additional chromosomes differ from the main one in the set of genes and specific features of replication; however, they carry genes vitally important for the bacterium. The role of these chromosomes and their probable origin from megaplasmids are discussed.

Key words: main and additional chromosomes, vitally important genes, structure of the region of replication origin, megaplasmids.

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Since the 1950–1960s and for a rather long time, it was a generally accepted concept in bacterial genetics that a bacterial cell contains a single chromosome (nucleoid). Since then, bacterial cytology and cytogenetics have achieved great successes. Especially fruitful proved to be the combination of electron, luminescent, and immunoluminescent microscopy with biochemical and genetic methods of studying replication of the bacterial chromosome. The most important result of these studies carried out on bacteria possessing one chromosome (primarily, *E. coli* and *B. subtilis*) was the discovery of the undoubtable similarity (although not identity) of the partitioning of replicated nucleoids with eukaryal mitoses (see reviews [1–3]).

Beginning with the late 1980s, data began to accumulate showing that bacteria of certain genera may contain more than one chromosome per cell. The additional chromosome is usually smaller than the main one (minichromosome) and has certain specific features. The research works devoted to microorganisms possessing a set of chromosomes are much less numerous than works dealing with "classical," one-chromosome bacteria. Nevertheless, such an organization pattern of the hereditary apparatus deserves attention, if only because in some bacteria, namely, vibrios, it occurs so widely that may be considered nearly a generic trait. It should be also noted that additional chromosomes have features in common with megaplasmids (there is a permanent problem with their differentiation), and, therefore, the studies of minichromosomes and plasmids constantly call to one another.

What should be considered an additional chromosome? Since the cell always contains other replicons, large and small, it is thought that, to be considered an additional chromosomes, a replicon should fit the following requirements [4]: it should be sufficiently large (about 400 kb or larger), but smaller that the main chromosome; as distinct from megaplasmids, which are similar in size, it should bear genes vitally important for the cell; and it should not be lost by the cell under any conditions. Other features which are typical of additional chromosomes but not necessary will be discussed later.

Not all of the above criteria are unambiguous. For example, the bacterium *Buchnera* sp. APS, an intercellular symbiont of aphids, possesses a small plasmid pLeu (7.8 kb), which carries leucine biosynthesis genes vitally important for the bacterium and the host; this plasmid is considered to be a chromosome [5]. Rhizobia, which are legume symbionts, have a complicated organization of the genome, which consists of a large circular chromosome and two megaplasmids [6]. The megaplasmids may be incorporated into the chromosome and may be split from it [7]. The larger megaplasmids bear genes that are vitally important for the bacterium, but only during its life in the free state in soil; therefore, only with reserve can it be considered an additional chromosome with (see below).

The methods most often used for studying additional chromosomes should be briefly discussed. Minichromosomes are sufficiently large replicons; therefore, determination of their size as a sum of restriction fragment lengths cannot involve frequently cutting restriction enzymes and separation in one-dimensional electrophoresis: too many merging bands would be produced, and the fragments larger than 50 kb would also be difficult to separate under these conditions. Therefore, the very first studies of minichromosomes involved infrequently cutting restriction endonucleases and pulsed-field electrophoresis, which allows very

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large linear DNA fragments to be separated. In order to determine whether these fragments carry vitally important genes, hybridization with corresponding DNA probes was carried out (usually with probes targeting such vitally important genes as rRNA genes).

In addition, pulsed-field electrophoresis was used for approximate determination of the number of large replicons in cell lysates obtained after in situ lysis of cells sealed in electrophoretic gel. This determination did not involve treatment with restriction endonucleases. Although large circular DNA molecules do not enter the gel, some of them have spontaneously arising breaks; therefore, each replicon yields a certain amount of linear molecules, which can be separated by pulsed-field electrophoresis. The number of bands formed corresponds to the number of different circular replicons present in the lysate (additional chromosomes, as well as megaplasmids, are usually circular).

Finally, sequencing of additional chromosomes is an important stage in their study. It yields complete data on the kinds of genes carried by them (including information on the vital importance of these genes). Moreover, the structure of the region of replication origin in additional chromosomes is different from that in the main chromosomes. The cytological picture of the segregation of additional chromosomes also has its specific features.

1. BACTERIA WITH ADDITIONAL CHROMOSOMES

Whether by chance or not, additional chromosomes have mainly been found in bacteria of the phylum *Proteobacteria.* It is expedient to group the relevant publications by the affiliation of bacteria possessing additional chromosomes with particular subgroups of this phylum. This will allow me to mention certain particularities of the way of life of the bacteria of the corresponding genera and species, which should be done because attempts have been made to relate these particularities with the complexity of the genomes. It should be emphasized here that bacteria with additional chromosomes constitute only a small portion of all proteobacteria.

1.1. Representatives of the Genus Rhodobacter, Brucellas, Agrobacteria, and Rhizobia (Class Alphaproteobacteria)

The first papers reporting the presence of two different chromosomes in a bacterial cell were published in 1989; these studies were performed with *Rhodobacter sphaeroides* 2.4.1 [8, 9]. This microorganism is a phototrophic bacterium capable of carbon dioxide fixation. The studied strain contained, in addition to the chromosomal genome, five large plasmids of various sizes (in total, the plasmid DNA length was 450 kb, about 10% of all cell DNA). Using a specially elaborated method that included treatment with infrequently cutting restriction endonucleases (so-called macrorestriction), use of cosmids, and Southern blot hybridization, the researchers managed to identify the DNA fragments obtained after macrorestriction and to determine their mutual disposition. They proved to be fragments of two circular chromosomes, measuring 3046 ± 95 kb and 914 ± 17 kb (chromosomes I and II, respectively). Chromosome I bore, in particular, one rRNA gene (*rrnA*); chromosome II bore two others (*rrnB* and *rrnC*). Both chromosomes contained genes of tRNA; chromosome II bore the *gapB* gene (vitally important gene responsible for synthesis of glyceraldehyde-3-phosphate dehydrogenase). The ratio of the chromosome contents in cells was always the same, 1 : 1. All other tested strains of *R. sphaeroides* also had two chromosomes per cell, although the set of plasmids and the number of replicons varied. All this, and especially the presence of vitally important genes, allowed the authors to state that chromosome II is indeed an independent chromosome, although not very large (by that moment, megaplasmids of similar or even larger size had already been described in some other bacteria). It should, however, be mentioned that no additional chromosomes were subsequently found in another *Rhodobacter* species, *R. capsulatum* [10].

Other papers of the same authors, published three years later [11, 12], reported genetic evidence of the chromosomal nature of both circular structures. At least some plasmids of *Rhodobacter sphaeroides* turned out to be self-transmissible (i.e., capable of conjugal transfer to other cells). Both chromosomes were lacking this capacity. However, the authors managed to construct a cassette that included *oriT* (the site of initiation of the so-called transfer replication) of self-transmissible plasmids, several genes of antibiotic resistance, and transposon *Tn5.* Due to the presence of the transposon, this cassette could be inserted into large and small chromosomes; the presence of the plasmid *oriT* provided for its transmissibility, and the presence of a set of antibiotic resistance genes allowed the dynamics of the transfer process to be monitored. The transfer of genes exhibited polarity (i.e., it occurred in a certain succession) and could be interrupted by intense shaking of the mixed suspension of donor and recipient cells. In this way, *R. sphaeroides* strains were obtained that were similar to *Hfr*-strains of *E. coli* and other bacteria capable of conjugation. The chromosomal status of the small second circular structure of the *R. sphaeroides* genome was finally proven. Further studies, devoted to genetic analysis of the small chromosome, showed that the two *R. sphaeroides* chromosomes are equivalent in terms of gene density and equally important in terms of gene composition [13, 14]. The concept that the two *R. sphaeroides* chromosomes are “equal partners” was also developed in paper [15].

After the studies on the structure of the *R. sphaeroides* genome, similar studies were performed with other alphaproteobacteria, brucellas first of all (*Brucella melitensis, Br. abortus, Br. suis, Br. ovis*). Brucellas are

small gram-negative pathogenic bacteria that are intracellular parasites and causative agents of various forms of brucellosis, which is a severe chronic disease in humans and animals. Taxonomically, brucellas are considered to be close to rhizobia and agrobacteria; it is possible that the ancestor of brucellas was a soil bacterium tending toward symbiosis with plants [16].

At first, it was reported that *Br. melitensis* has only one circular chromosome 1.3 Mb in size [17]. However, two years later a paper was published demonstrating the existence in this bacterium of two circular replicons sized 2100 and 1150 kb [18]. This study was performed according to the same scheme as the studies with other bacteria mentioned above. This scheme involved cleavage with infrequently cutting restriction endonucleases, Southern hybridization with the aim to detect certain genes on replicon fragments, and pulsed-field electrophoresis of native DNA from cell lysate. rRNA genes were detected on both replicons; the smaller replicon also contained some other vitally important genes, and this provided grounds to consider it an additional chromosome rather than a megaplasmid. Pulsed-field electrophoresis of native DNA of *Br. abortus, Br. suis,* and *Br. ovis* also yielded two bands, corresponding to two chromosomes.

Additional information was provided by complete sequencing of the genomes of *Br. suis* and *Br. melitensis* [16, 19]. The chromosomes of *Br. suis* were sized 2107 and 1207 kb. The larger replicon was a typical bacterial chromosome with a cluster of genes *dnaA, dnaN, recF* in the region of replication origin. On the contrary, the gene cluster in the region of replication origin in the smaller chromosome was similar to that of plasmids (including a gene similar to the plasmid gene of replication initiation protein RecC, and genes of proteins RepA and RepB, responsible for plasmid distribution between dividing cells in Ti plasmids and plasmids of rhizobia. Pronounced asymmetry was observed between the compositions of genes in the larger and smaller chromosomes. In the larger chromosome, most genes of transcription and translation were located; specifically, it encoded 51 of the 53 ribosomal proteins and 41 of 55 tRNAs. In the smaller chromosome, genes of membrane transport and energy metabolism were "excessively" represented. However, even the few genes of ribosomal proteins and tRNAs located in the smaller replicon indicated its chromosomal nature.

Interestingly, one of four other biovars of *Br. suis* possessed only one chromosome, sized 3.3 Mb [20]. This fact prompted one of the speculations on the origin of additional chromosomes in bacteria (see below).

Additional chromosomes were also found in alphaproteobacteria pathogenic for plants (*Agrobacterium tumefaciens, A. rubi*, and other species and subspecies) [21]. In strain *Agrobacterium tumefaciens* C58, pulsed-field electrophoresis revealed four replicons: a Ti plasmid, a cryptic megaplasmid, and two very large DNA molecules (3000 and 2100 kb). The larger molecule was circular, and the smaller, additional one, was linear. Both chromosomes bore vitally important genes of rRNA and genes of protein synthesis. These conclusions were on the whole confirmed in later studies of several *A. tumefaciens* biovars and other agrobacteria [22].

The genome of *A. tumefaciens* C58 was later completely sequenced in two independent studies, which produced identical results [23, 24]. The sizes of this complex genome were accurately determined; it included a circular chromosome (2800 kb), a linear chromosome (2075 kb), and two plasmids: pTiC58, inducing plant tumor (214 kb), and pAtC58 (543 kb), which was cryptic. The main, circular chromosome and the linear one each contained two rRNA operons and tRNA genes (40 in the circular one and 13 in the linear). On the whole, gene "density" was the same in the two chromosomes; however, some genes were unevenly distributed (thus, the main chromosome contained 75% of genes of nucleotide metabolism, and the additional chromosome contained only 25%). At the same time, genes of lipid metabolism were represented in equal proportions. The most pronounced were the distinctions in the regions of replication origin: in the circular chromosome, organization of this region was bacterial-type, and in the linear chromosome and in the other two replicons, it was of the so-called *repABC* type, peculiar to some plasmids (see below).

One more study [25] employed cytological methods, namely, selective staining with luminescent dyes (so-called FISH) of particular chromosome sites, with the aim to determine the mutual disposition in the *A. tumefaciens* cells of replication origin regions of the main and additional chromosomes. 68% coincidence of luminescent dots (corresponding to replication origins of the two chromosomes) was observed, suggesting that the localization of the origins in the cell was the same. As will be seen below, similar experiments with the main and additional chromosomes of vibrios produced different results.

As early as in 1990s, three very large circular replicons were found in rhizobia, which are legume symbionts capable of thriving both in nodules formed on plant roots and in a free-living state in soil [6, 26]. One of this replicons, sized more than 3500 kb, was a chromosome by all criteria; the two other ones were for a long time considered to be megaplasmids. Ten years later, data were published on the complete sequence of all replicons of *Sinorhizobium meliloti*, a lucern symbiont [27–29]. The largest replicon was a chromosome sized 3650 kb. In addition, the bacterium possessed the replicons *pSymB* (1700 kb) and *pSymA* (1350 kb). *pSymA* had all characteristics of a megaplasmid: it lacked vitally important genes, had a structure of the replication origin region typical of plasmids, and exhibited self-transmissibility. The G+C content of *pSymA* differed from that of the main chromosome. *pSymB* contained the vitally important genes *minCDE* (their

products are involved in cell division) and genes responsible for the synthesis of asparagine synthetase. This provided grounds to consider *pSymB* an additional chromosome, although most of its genes were related to existence of the bacterium in soil as a saprophyte. The authors of the papers cited kept for this replicon the plasmid name *pSymB*, making a cautious conclusion that *pSymA* has evident plasmid features and *pSymB* has certain chromosomal traits [27].

1.2. Burkholderias (Class Betaproteobacteia)

Burkholderias were earlier assigned to the genus *Pseudomonas.* The first representative of these bacteria that was tested for the presence of additional chromosomes in the early 1990s bore at that time the name *Pseudomonas cepacia* 17616 [30]. Later, these bacteria were redescribed as a separate genus *Burkholderia*, belonging to betaproteobacteria.

Burkholderias are soil bacteria. Some of them are plant pathogens (e.g., *Bh. cepacia* causes onion rot); they may be conditionally pathogenic for humans (the aforementioned *Bh. cepacia* causes complications in patients with fibrinous cystitis). *Bh. mallei* is the causative agent of glanders, a very dangerous infection of animals and humans; *Bh. pseudomallei* causes glanders-like diseases and septicemia.

In *Bh. cepacia* 17616, several large circular replicons were detected by traditional methods; three of them carried rRNA genes. The sizes of these replicons were 3400, 2500, and 900 kb. Judging from the presence of vitally important genes, all three of these replicons are chromosomes [30]. In a subsequent work [31], 11 more strains belonging to the same species were studied (some of them most probably represented subspecies). All of them had more than one chromosome (from two to four). The total size of the genome reached 8000 kb. The strains also bore large circular plasmids. The authors of [31] and other [32, 33] papers tried to relate the complexity of the *Bh. cepacia* genome to the outstanding versatility of the metabolism of this bacterium, which can degrade many chemical compounds and may thrive as a saprophyte, commensal, or parasite.

The genomes of *Bh. mallei* and *Bh. pseudomallei* have been completely sequenced [35, 36]. Both species proved to contain two chromosomes (3510 and 2320 kb in *Bh. mallei* and 4070 and 3170 kb in *Bh. pseudomallei*). The larger chromosomes had typical chromosomal features (the *dnaA* and *dnaN* genes in the region of replication origin). The smaller chromosomes, as in some other bacteria, were similar to plasmids of the so-called iteron type in the organization pattern of their regions of replication origin. Both larger and smaller chromosomes bore vitally important genes. Larger chromosomes contained many orthologous genes.

1.3. Vibrios (Class Gammaproteobacteria)

The genus *Vibrio* comprises over 60 species [37]. These are gram-negative halophilic bacteria inhabiting fresh and saline water; the genus also contains some pathogenic species, such as *V. cholerae*, the causative agent of "classical" cholera, and related species and subspecies, also causing acute gastrointestinal diseases, albeit with lower lethality. Vibrios can also cause diseases of fish and other aquatic animals. In most of the studies discussed below, *V. cholerae* was used as the object. This organism is capable of "double life" as either a free-living saprophyte, which can grow in aquatic environments with adequate temperature, probably due to decomposition of chitin-containing plankton, or as a causative agent of one of the most dangerous diseases, primarily affecting the human small intestine. Attempts have been made to relate some peculiarities of the cholera vibrio genome to this duality (see below).

The first works on the chromosome composition of vibrios were published, most likely independently, in 1998 and 1999 [38, 39]. In [38], data was presented concerning the genome composition of cholera vibrios (Ogawa type strain, several strains isolated from patients, and El Tor vibrio). In [39] and [40], a later publication of the same authors, information was provided on the genome composition of four *V. parahaemolyticus* strains (a gastroenteritis-causing vibrio; infection occurs via fish products). In both works the native, nonrestricted chromosomal DNA was separated by pulsed-field electrophoresis; the absence of plasmids in the vibrios under study facilitated the experiments. Two bands were revealed, belonging probably to two large structures. The DNA from each band was then treated with infrequently cutting restriction endonucleases; Southern hybridization was used for the subsequent identification of the fragments thus obtained. Both DNA bands corresponded to two circular structures with sizes of 2400 and 1600 kb for *Vibrio cholerae* and 3200 and 1900 kb for *V. parahaemolyticus.* Vitally important genes, including those encoding rRNA and heat shock protein, were present on both (large and small) circular structures. A conclusion was made that, rather than a chromosome and a megaplasmid, two chromosomes (large and small) were present in these vibrios. Although this conclusion was in contradiction with previous data indicating the presence of only one chromosome in cholera vibrio [41], it was readily accepted since other bacteria with more than one chromosome were already known. Moreover, the smaller circular structure satisfied the requirements to a chromosome listed in [4]: the presence of vitally important genes, considerable size, albeit less than that of the major chromosome, the absence of self-transmissibility, and the impossibility of being cured, i.e., persistent presence in the cell.

The presence of two chromosomes was demonstrated for 34 *Vibrio* species by pulsed-field electrophoresis of nonrestricted DNA, as well as for *Photo-*

bacterium and *Salinivibrio*, closely related to the genus *Vibrio* [42]. The work on complete sequencing of *V. vulnificus* also demonstrated the presence of two chromosomes [43]. While the larger chromosomes of all these microorganisms were of about the same size (3000 to 3300 kb), the size of the smaller chromosomes varied significantly (from 800 to 2400 kb). The variability of smaller chromosome size in vibrios has also been demonstrated in an earlier work [40]. Thus, the presence of two chromosomes was a persistent feature in all the vibrios investigated.

Complete sequencing of the genome of *V. cholerae* El Tor [44] provided highly important data concerning the gene composition of both chromosomes. One article [45] characterized these data as a treasure trove for the researchers planning to study the chromosome replication and segregation in cholera vibrios. The larger chromosome had a size of 2900 kb, the smaller one, 1070 kb (with G+C base contents of 47.7 and 46.9 mol%, respectively). The larger chromosome had a higher content of genes with known functions than the smaller one (58 and 41%, respectively). Although all kinds of genes were represented on both circular structures, their presentation on the larger chromosome was more complete. It contained most of the vitally important genes responsible for DNA replication, transcription, and translation, as well as for cell wall synthesis. However, a number of significant genes, including the genes encoding some ribosomal proteins, was located on the smaller chromosome as well; some genes (encoding proteins L20 and L35) were located only on the smaller chromosome. The genes important for survival in aquatic environments were located on both chromosomes (those encoding chitinase required for decomposition of the zooplankton exoskeleton and the quorum sensing genes). Apart from the genes encoding ribosomal proteins, certain other genes were also doubled, including some tRNA genes. The proteins encoded by the genes on the larger chromosome exhibited higher homology to the proteins of other *Gammaproteobacteria* than the same proteins encoded by the smaller chromosome genes.

The smaller chromosome was in some respects similar to megaplasmids. For example, the structure of its origin region was different from that of bacterial chromosomes. The genes located in the origin region of the larger chromosome were the same as in many other bacteria (*dnaA, dnaN, recF, gyrA*). In the smaller chromosome, the picture around the origin was quite different and resembled that known for some plasmids. The ParA protein corresponding to the *parA* gene of the larger chromosome (significant for postreplication segregation of chromosomes) was similar to such proteins in other bacteria; the ParA protein of the smaller chromosome, however, was more similar to the segregation proteins of plasmids and megaplasmids. Most interestingly, the smaller chromosome contained a region of considerable size (125.3 kb) with a structure typical for the so-called integrons of plasmids. The transposase genes and the host addiction genes important for the coexistence of bacteria and plasmids should be mentioned among the genes identified in this region.

These findings supported the hypothesis of the vibrio smaller chromosome being originally a megaplasmid captured by the *Vibrio* ancestors relatively long ago (see below). The mechanisms of this capture are completely unknown; traces of the former autonomy still remain, as was confirmed by later research on the replication of both chromosomes.

In this series of publications [46–49], so-called minichromosomes were constructed (not to be confused with additional chromosomes!), with the replication origins (*oriC*) from the large and small chromosomes of *V. cholerae* and other vibrios. These origins were incorporated into plasmids capable of mobilization but not of independent replication. Such minichromosomes were introduced into the cells of various *E. coli* strains and their replication (occurring due to the presence of the origins from vibrios) was investigated. Important structural and functional features of the origins of the large and small chromosomes were determined. In the larger chromosome, *OriC* had all the characteristics of gammaproteobacterial origins, including DNA boxes (sites for binding DnaA, the replication initiator of typical bacterial chromosomes), the binding site for the Dam protein (DNA adenyl methyl transferase), and the INF protein (stimulator of prereplication unwinding of DNA); its A + T content was high. *OriC* of the larger chromosome exhibited 58% identity with *E. coli OriC*. In the smaller chromosome, *OriC* was structurally different. Although some of its features were similar to the *OriC* of the larger chromosome (the DnaA and INF binding sites), it contained repeats typical of the origins of some plasmids, but not chromosomes: 11-nucleotide (four repeats) and 12-nucleotide (seven repeats). These repeats are typical of the origins of the so-called iteron plasmids (see review [50]). Furthermore, two genes (*rctA* and *rctB*) were flanking *OriC* in the smaller chromosome. The product of *rctB* is a polyfunctional DNA-binding protein with no analog among bacteria. It acts as a replication initiator of the smaller chromosome, but not of the larger one (protein DnaA also plays its part in the replication of the smaller chromosome), has autorepressor properties, and acts as a repressor of the *rctA* gene. The *rctA* product is not a protein but a nontranslated RNA, which is necessary for the replication of the smaller chromosome. In this respect, the smaller chromosome is similar to some plasmids. The mechanisms of initiation, however, are not clear (this RNA probably acts as a primer).

Thus, the structure and functioning of *OriC* of the smaller chromosome differ from the those of typical origin sites of bacterial chromosomes in at least three important respects: it contains unusual repeats and initiation of its replication requires RctB and RNA encoded by *rctA*. Moreover, components in common with the major chromosome (the DnaA protein and

adenyl methyl transferase) are required for replication. The issues of the replication times of both chromosomes and of coordination between their replication with the cell cycle were to be studied.

In one of the relevant works [51], the correlation between the cell cycle and chromosome replication was investigated. As mentioned above, the smaller chromosome resembles a megaplasmid in some of its characteristics; although the distribution of large plasmids in dividing cells is regular, it is less closely associated with the distribution of chromosomes (nucleoids). The F plasmid, for example, may be distributed, after replication, even among nucleoid-free mutant cells [52]. Flow cytometry and introduction of light and heavy isotopic labels into the vibrio DNA revealed that both chromosomes were replicated synchronously with the events of the cell cycle (i.e., with cell growth and division), and only once during the cell generation time. It was therefore concluded that the smaller chromosome behaved as a chromosome rather than as a megaplasmid. In another work [53], cytological techniques were used in order to determine the behavior of the two origins of cholera vibrio chromosomes within the cell space. Two different gene cassettes able to combine with fluorescent dyes were inserted into the *OriC* sites. Two luminous points of different color were readily revealed by microscopy (additional staining enabled visualization of the chromosome "body" as well). The movement of the chromosome origins indicated their different behavior. Both luminous points were superimposed only in 3% of the cells. The origin of the larger chromosome in young cells (immediately after division) was closer to one of the cell poles; in the course of replication, one of the luminous points moved closer to the pole, while the other migrated in the opposite direction. The origin of the smaller chromosome was located in the center of the cell; after replication, the origins moved symmetrically to the poles of a dividing cell. The origin of the smaller chromosome commenced duplication later than the origin of the larger one. In a recently published paper [54], a delay in the replication of the smaller chromosome was also reported. In this work, beginning of the replication of the smaller chromosome was detected when the replication of the larger chromosome was close to its end; however, replication of the two chromosomes culminated simultaneously.

The complex hierarchy of control of the replication of the vibrio smaller chromosomes is usually attributed to the fact that the presence of two initiators removes the risk of exhaustion of one of them [48]. The same principle of initiator economy may be used to explain deleted replication of the smaller chromosome. In vibrios, the cell cycle is very short (approximately 10 min); this implies additional requirements on the accuracy of regulation of all cellular processes.

1.4. Leptospiras (Phylum Spirochaetes)

Leptospira, belonging to phylum *Spirochaetes*, is the only genus beyond the phylum *Proteobacteria* for which presence of two chromosomes in the cell has been definitely established. Leptospiras may thrive in water bodies, where they arrive with rat urine; however, they are facultative parasites causing leptospirosis in humans, with affection of liver and kidney tissues and vast hemorrhages in other organs. In two serovars of *Leptospira interrogans*, two large circular replicons were found, considerably differing in size (4500 and 350 kb) [55]. The larger replicon bore *dnaA* and *gyrAB* genes, typical of the bacterial region of replication origin, and many other vitally important genes. The smaller replicon bore some genes necessary for protein synthesis, which allowed it, in spite of its tenfold smaller size, to be considered an additional chromosome.

Later, the complete genome sequence of another serovar of *L. interrogans* (serovar *Icterohaemorrhagiae*) was determined [56]. Two replicons, 4330 and 359 kb in size, were found, and both were chromosomes. They had the same G+C content. The larger chromosome bore all genes of ribosomal and transport RNA, and many other vitally important genes. The smaller chromosome also contained some vitally important genes (genes of amino acid synthesis, NADH dehydrogenase genes, etc.). Thus, different serovars of leptospiras had similar genome structures.

2. MEGAPLASMIDS IN THE PAST, MINICHROMOSOMES IN THE PRESENT?

When discussing the nature of additional chromosomes in bacteria, two interrelated questions usually arise that concern the origin of additional chromosomes and their role in the cell. Two hypotheses exist about the origin of minichromosomes (see review [57]). The first of them, the so-called plasmid hypothesis, is more substantiated. According to this hypothesis, additional chromosomes once were megaplasmids, which arrived in the cell as a result of horizontal transfer. At that time, they did not bear genes vitally important for the cell. In the endless succession of generations, adjustment of the chromosomal and plasmid replicons occurred, accompanied by numerous crossing-over events; as a result, the megaplasmid acquired typical chromosomal genes, including copies of rRNA genes (however, on the whole, the individuality of the gene sets in both replicons was retained). In parallel, amelioration of the externally acquired genes occurred: the foreign DNA gradually acquired, under mutational pressure resulting from operation of DNA polymerases, repair systems, etc., a G+C content peculiar to the host chromosome [58]. As a result of amelioration, the G+C content of the minichromosomes became almost the same as that of the main chromosomes. However, the origination of

minichromosomes from plasmids is evidenced by the structure peculiarities of the region of replication origin.

In the main bacterial chromosomes, the indispensable components of this region are the *dnaA, dnaN, recR*, and *gyrA* genes, as well as specific repeats (see reviews [59, 60]). In additional chromosomes, the region of replication origin has a different structure (see section 1.3 above, devoted to the chromosomes of vibrios), exhibiting features in common with the corresponding region of iteron-type teta plasmids (e.g., P1, F, R1). In these plasmids, the region of replication origin contains direct repeats of a particular composition, so-called iterons, which are the sites of interaction with RepA, the protein of initiation of plasmid replication. Both iterons and protein RepA differ from DNA boxes and DnaA protein of the bacterial chromosome [50, 61]. In addition, most of these plasmids encode proteins RepB and RepC, necessary for accurate distribution of replicated plasmids between bacterial cells. Plasmids that have all these features are sometimes called ABC-type plasmids.

Certain details of replication of additional chromosomes and their segregation between daughter cells also differ, at least in vibrios, from those peculiar to the main chromosomes. However, it is noteworthy that the replication mechanisms of both chromosomes are coordinated with one another and with the cellular cycle. Thus, minichromosomes, despite all their peculiarities, obey the laws governing the life of the cell.

The second hypothesis attempting to explain the origin of additional chromosomes in bacteria is called the schism hypothesis. This hypothesis implies that the ancestral chromosome was once doubled and split, as a result of crossing over, a large fragment which bore, in particular, the region of replication origin [62]. Such a fragment could have become a small independent chromosome. Such a way of origination of the minichromosome agrees with certain observations that hardly fit the plasmid hypothesis. Thus, it was mentioned above that, in *A. tumefaciens*, as distinct from vibrios, the regions of the replication origin in the large and small chromosomes were located, as shown by immunoluminescent microscopy, in one and the same point in the cell [25]. One of the four serovars of *Br. suis* possesses a single chromosome [20], which may be a result of recombination between homologous regions in the main and additional chromosomes with return to the initial, single-chromosome state. However, the schism hypothesis fails to explain differences in the structure of the regions of replication origin and in the replication processes of the two chromosomes; neither can it explain the fact that minichromosomes are poor in vitally important genes (the minichromosome of *R. fascians* may probably be considered an exception). Thus, the plasmid hypothesis seems more plausible, at least as applied to additional chromosomes of vibrios.

However, the plasmid hypothesis poses several questions that cannot be answered unequivocally. Since the bacterial cell can hardly be indifferent to the presence of an additional chromosome, it has to be assumed that this replicon is useful to it. The usefulness of large conjugative plasmids and megaplasmids for symbiotic bacteria has long been implied; but what are the benefits from the transformation of a megaplasmid into a minichromosome (if such a transformation in fact occurs)? Why are additional chromosomes (megaplasmids in the past) not incorporated into the main chromosome with the formation of a combined genetic structure, as are incorporated other DNA fragments upon horizontal transfer? The answers that have been given to these questions are rather obscure; thus, it has been supposed that presence of two chromosomes (not just replicons, but chromosomes) increases the plasticity of the genome, and that doubling of some vitally important genes provides a safety margin for the genome. It has been stated that it is not by chance that additional chromosomes are characteristic of bacteria that can exist both as saprophytes and parasites. In *V. cholerae*, whose minichromosome has been most thoroughly studied, its presence has also been related to hypothetical advantages provided by this state of genome to bacteria capable of thriving in two different ecological niches (*V. cholerae* can develop in water bodies, decomposing with chitinases the exoskeleton of planktonic animals, and in humans, attacking the cells lining the intestine). The transcription of both chromosomes of the cholera vibrio has been studied under different growth conditions (growth in liquid nutrient medium and cultivation in ligated rabbit intestinal loop [63]). It was shown that genes of the main chromosome were transcribed equally efficiently under both cultivation conditions. Many of the genes of the smaller chromosome were transcribed only in the intestine. The supposition was made that the role of some genes of the smaller chromosome (which are not vitally important) consists in increasing the viability of the bacterium under conditions of the intestine [42]. It is however difficult to understand why these genes could not play the same role located in a single united chromosome, whose replication would not be complicated by the necessity of coordinating with the replication of another chromosome. In one of the papers concerning this issue, the remark is made, which I consider appropriate, that it is tantalizing to speculate how coordinated chromosome replication influences *V. cholerae* survival (p. 1134).

It is evidently premature to put forward experimentally supported hypotheses as to why it is sometimes beneficial for bacteria to have two and not one chromosome. Unfortunately, experimental tests of such hypotheses are hindered by the impossibility of curing the cell of the additional chromosome. The only paper on a *Br. suis* serovar that lacked an additional chromosome [20] contains no data on any other differences between this serovar and serovars possessing two chromosomes. Probably, it is not reasonable to search for expediency underlying each biological phenomenon.

However, the desire to do so is an intrinsic feature of human beings, researchers in particular.

Of the publications not yet mentioned in this review, I would like to draw the readers' attention to two reviews [64, 65] and the experimental paper by Srivastava et al. [66].

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