



Influence of Origin Recognition Complex Proteins on the Copy Numbers of Three Chromosomes in *Haloferax volcanii*

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ABSTRACT Replication initiation in archaea involves a protein named ORC, Cdc6, or ORC1/Cdc6, which is homologous to the eukaryotic origin recognition complex (ORC) proteins and to the eukaryotic Cdc6. Archaeal replication origins are comprised of origin repeat regions and adjacent *orc* genes. Some archaea contain a single replication origin and a single *orc* gene, while others have more than one of each. *Haloferax volcanii* is exceptional because it contains, in total, six replication origins on three chromosomes and 16 *orc* genes. Phylogenetic trees were constructed that showed that *orc* gene duplications occurred at very different times in evolution. To unravel the influence of the ORC proteins on chromosome copy number and cellular fitness, it was attempted to generate deletion mutants of all 16 genes. A total of 12 single-gene deletion mutants could be generated, and only three *orc* gene turned out to be essential. For one gene, the deletion analysis failed. Growth analyses revealed that no deletion mutant had a growth defect, but some had a slight growth advantage compared to the wild type. Quantification of the chromosome copy numbers in the deletion mutants showed that all 12 ORC proteins influenced the copy numbers of one, two, or all three chromosomes. The lack of an ORC led to an increase or decrease of chromosome copy number. Therefore, chromosome copy numbers in *Hfx. volcanii* are regulated by an intricate network of ORC proteins. This is in contrast to other archaea, in which ORC proteins typically bind specifically to the adjacent origin.

IMPORTANCE The core origins of archaea are comprised of a repeat region and an adjacent gene for an origin recognition complex (ORC) protein, which is homologous to eukaryotic ORC proteins. *Haloferax volcanii* is exceptional because it contains six replication origins on three chromosomes and an additional 10 *orc* genes that are not adjacent to an origin. This unique ORC protein repertoire was used to unravel the importance of core origin *orc* genes and of origin-remote *orc* genes. Remarkably, all ORC proteins influenced the copy number of at least one chromosome. Some of them influenced those of all three chromosomes, showing that cross-regulation in *trans* exists in *Hfx. volcanii*. Furthermore, the evolution of the archaeal ORC protein family was analyzed.

KEYWORDS Archaea, *Haloferax volcanii*, ORC proteins, deletion mutants, essentiality, origin recognition complex, phylogenetic analysis, polyploidy, replication origin

The archaeal replication machinery is homologous to that of its eukaryotic counterpart, not to the bacterial replication machinery. However, while eukaryotes contain more replication proteins and/or more paralogs of replication proteins, archaea contain a simpler version of the replication machinery (1). In eukaryotes, replication is initiated by the binding of a heteromeric origin recognition complex (ORC) to replication origins. The ORC is comprised of six subunits, ORC1 to ORC6 (2). With the help of the proteins Cdc6 and Cdt1, it recruits the minichromosome maintenance protein (MCM) complex to the origin, which is the replicative helicase. The MCM complex is also a heteromeric

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complex comprised of six subunits, MCM2 to MCM7 (2). The eukaryotic ORC has regulatory functions beyond replication initiation, and its malfunction has been connected to the development of various human diseases (3). Very surprisingly, in contrast to the high importance of the ORC, it has recently been found that two of the human *orc* genes are not essential (4).

Archaea contain a replication initiation protein that is homologous to the ORC proteins and has its highest similarity to ORC1. It is also homologous to Cdc6, and therefore, it is often termed "ORC1/Cdc6." However, also the names ORC1 and Cdc6 are commonly used. For the sake of simplicity, we will call the archaeal initiation protein ORC in this paper. It was previously shown that in *Sulfolobus*, the ORC protein fulfills both functions of the eukaryotic ORC and the Cdc6 protein, i.e., binding to the origin and recruitment of the replicative helicase, MCM (5).

Eukaryotes typically contain thousands of replication origins. In stark contrast, bacteria contain only one origin of replication and one replication terminus at opposite sites of a circular chromosome. Some archaea also contain one origin of replication (6). However, it has been shown that various species of archaea contain more than one replication origin, and thus multiorigin replication seems to have evolved in archaea. A recent review gives an excellent overview of the diversity of replication in *Archaea* (7). The initial observation was that two species of *Sulfolobus* contain three replication origins (8), but subsequently, 2 to 4 replication origins on the main chromosome were also found in *Pyrobaculum calidifontis* (9) and in several species of haloarchaea (10, 35). Archaeal replication origins are comprised of (i) a noncoding region containing AT-rich DNA unwinding elements (DUEs), which are important for DNA melting, and origin recognition boxes (ORBs), and (ii) an adjacent *orc* gene. For *Haloarcula hispanica*, it has been shown that the origins are specifically dependent on the respective colocalized *orc* gene (11). Also, for *Sulfolobus*, it has been shown that each of the three replication origins is addressed by a specialized replication initiation protein, two by an ORC paralog and one by a Cdt1 homolog (12). Typically, the number of replication origins and ORC proteins in archaea is identical, e.g., *Pyrococcus* has a single replication origin and a single *orc* gene (13). However, the situation is very different in haloarchaea, which typically contain more *orc* genes than replication origins. For example, *Haloferax volcanii* contains six replication origins, three on the major chromosome and one each on three minor chromosomes (14). In the commonly used laboratory strain *Hfx. volcanii* H26, one of the three minor chromosomes is integrated into the major chromosome, and H26 thus contains three replicons, one with four replication origins and two with one replication origin each (14). In contrast, the *Hfx. volcanii* genome contains 16 *orc* genes, and thus the majority of 10 *orc* genes are not located adjacent to origin repeats (www.halolex.mpg.de) (15). Another feature of *Hfx. volcanii* is that it is polyploid and contains about 20 copies of the major chromosome (16). The chromosome copy number can vary widely and can range from two to more than 40 (17). The copy number regulation is related to the number of replication initiation events per cell cycle. During steady state, each origin must fire exactly once per cell cycle, irrespective of the chromosome copy number. However, more than one replication event per cell cycle is needed to increase the copy number, and less than one initiation event per cell cycle results in a decrease of copy number. Therefore, several or all of the ORC proteins might be involved in chromosome copy number regulation. To test this hypothesis, all 16 *orc* genes were chosen to construct single-gene deletion mutants. The effects of the nonessential *orc* gene deletions on the copy numbers of all three chromosomes and on the fitness of the cells were quantified. Furthermore, phylogenetic trees were constructed to analyze the evolution of the ORC protein family.

RESULTS AND DISCUSSION

Evolution of the *Hfx. volcanii* ORC proteins. To unravel the evolution of the ORC protein family, the ORC proteins of five haloarchaea (representing five different genera), of two methanogenic archaea, and of five additional euryarchaeota and crenarchaeota were chosen, and phylogenetic trees were generated. Figure 1 shows the consensus

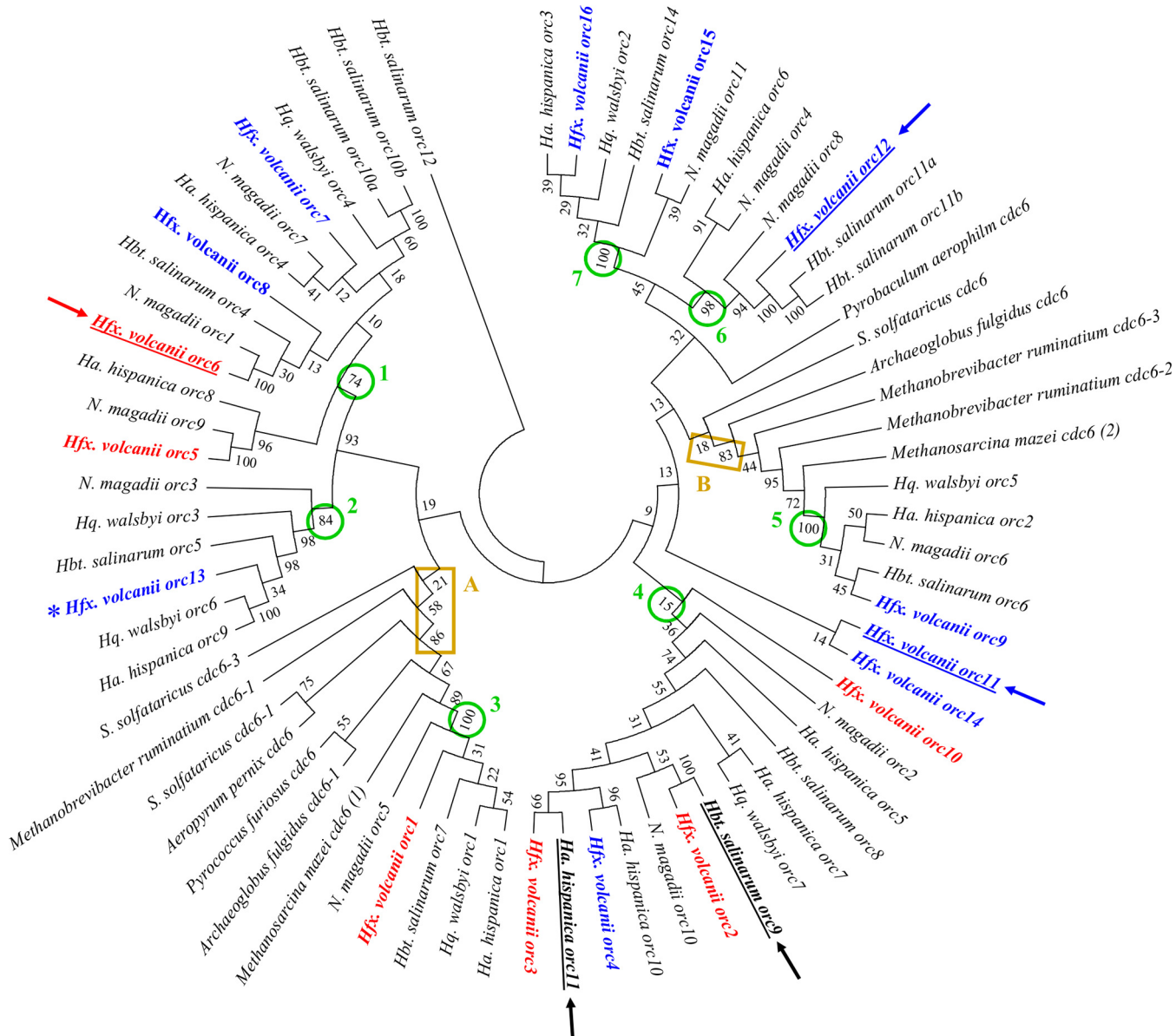


FIG 1 Phylogenetic tree of the ORC protein family. A consensus tree that was generated by neighbor joining after 1,000 bootstrap replications is shown. The bootstrap values (percent) are indicated at the respective nodes. The 12 ORC proteins of this study are colored as follows: the six proteins encoded adjacent to origin repeats are shown in red, and the 12 proteins encoded elsewhere are shown in blue. The essential ORC proteins of *Hfx. volcanii*, *Hbt. salinarum*, and *Ha. hispanica* are underlined and marked with an arrow. The seven nodes that define ORC paralogs that were already present in the common ancestor of *Hfx. volcanii*, *Hbt. salinarum*, *Ha. hispanica*, and *N. magadii* are marked by a circle and are numbered. The nodes that define two ORC paralogs that predated the evolution of the *Halobacteriaceae* are boxed. The *orc* gene that could not be analyzed is marked with an asterisk.

tree obtained after 1,000 bootstrap replications using the neighbor-joining approach. A very similar tree was obtained after 1,000 bootstrap replications using the maximal parsimony algorithm (data not shown). The tree revealed that the last common ancestor of *Hfx. volcanii*, *Halobacterium salinarum*, *Haloarcula hispanica*, *Haloquadratum walsbyi*, and *Natrialba magadii* already contained seven ORC proteins. The seven nodes are indicated in Fig. 1. In six cases, the monophyletic groups defined by the nodes contain ORC paralogs of all five species; only group six contains paralogs of only four species, and thus it seems that this *orc* gene has been secondarily lost in *Haloquadratum walsbyi*. The tree shows that the multiplication and diversification of the *orc* genes is not a recent event in the genus *Haloferax* but started early in the evolution of the *Halobacteriaceae*. Node 3 contains the highly conserved ORC1 protein, which is or-

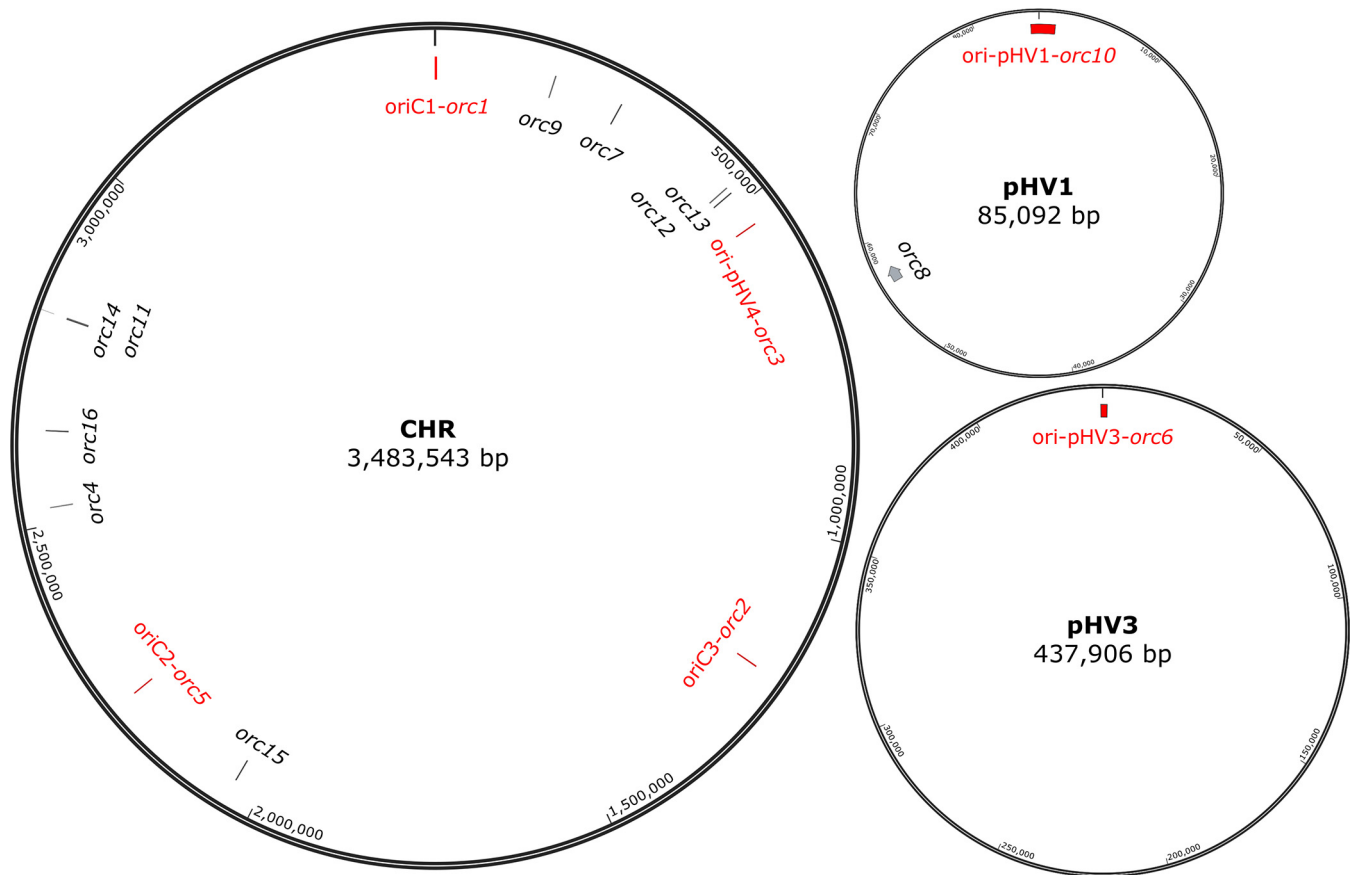


FIG 2 Schematic overview of the three replicons and the localizations of the 16 *orc* genes of *Hfx. volcanii*. Names and sizes of the replicons are included; the circles are not drawn to scale.

thologous to ORC proteins from methanogenic archaea and other euryarchaeota and crenarchaeota (node A in Fig. 1). Thus, the class of node A ORC proteins seems to be the most ancient, because it is widely distributed in many groups of archaea. There is a second family of ORC paralogs that contains proteins from other euryarchaeota, indicating that the presence of two ORC proteins predates the evolution of the *Halobacteriaceae* (node B in Fig. 1).

The evolution of the seven ORC subfamilies was very diverse; the node 3 and 5 subfamilies contain only a single sequence of the five haloarchaeal species, and thus no further gene duplication occurred. In stark contrast, the node 4 subfamily contains four sequences each of *Hfx. volcanii* and *Har. hispanica*, two each of *Hbt. salinarum* and of *N. magadii*, and only one of *Hqu. walsbyi*. The node 7 subfamily contains two sequences of *Hfx. volcanii* and one sequence each of the four other species and thus gives evidence for a gene duplication event that happened late and only in the evolution of *Hfx. volcanii*. Taken together, the evolution of the ORC protein family is very complex, and the 16 *orc* genes of *Hfx. volcanii* evolved through gene duplications that happened at very different times in its evolution.

Analysis of essentiality of all 16 *orc* genes. The localization of the 16 *orc* genes on the three chromosomes is schematically shown in Fig. 2; the origin-adjacent *orc* genes are shown in red. The minor chromosome pHV3 is essential because it encodes, e.g., the enzymes for nitrate and dimethyl sulfoxide (DMSO) respiration, the degradation pathways for xylose and arabinose, many ABC transporters, translation initiation factor aIF1, and transfer-messenger RNA (tmRNA). It is not clear whether the minor chromosome pHV1 is essential. It contains only 89 protein-coding genes, many of which encode conserved hypothetical proteins. However, it also contains an ABC transporter operon, which might be essential under specific conditions.

TABLE 1 Summary of *orc* genes in *Hfx. volcanii*

Replicon	Gene no.	Gene name	Genomic region/strand deleted region ^e	Gene length (bp)	Avg coverage (×) ^f
Chr	HVO_0001 ^d (ori1) ^a	<i>orc1</i> ^d	258–1952/F 282–1925	1,695	ND
Chr	HVO_0194 ^a	<i>orc9</i>	174892–176016/F 174909–176000	1,125	1,116
Chr	HVO_0634 ^d (ori3) ^a	<i>orc2</i> ^d	569203–570429/R 569215–570384	1,227	252
Chr	HVO_1537 ^a	<i>orc15</i>	1403626–1404828/R 1403674–1404810	1,203	40
Chr	HVO_1725 ^d (ori2) ^a	<i>orc5</i> ^d	1594707–1595912/F 1594719–1595900	1206	50
Chr	HVO_2042 ^a	<i>orc4</i>	1888354–1889583/F 1888369–1889568	1,230	102
Chr	HVO_2133 ^a	<i>orc16</i>	1998477–1999643/R 1998492–1999634	1,167	10
Chr	HVO_2292 ^a	<i>orc14</i>	2160851–2161873/R 2160870–2161857	1,023	ND
Chr	HVO_2293 ^b	<i>orc11</i>	2162076–2162873/F	798	ND
pHV4	HVO_A0001 ^d (ori-pHV4) ^a	<i>orc3</i> ^d	152–1402/F 167–1387	1,251	62
pHV4	HVO_A0064 ^c	<i>orc13</i>	55398–56897/R	1,500	55
pHV4	HVO_A0072 ^b	<i>orc12</i>	65261–66289/R	1,029	ND
pHV4	HVO_A0257 ^a	<i>orc7</i>	257391–258668/R 257415–258656	1,278	171
pHV3	HVO_B0001 ^d (ori-pHV3) ^b	<i>orc6</i> ^d	201–1433/F 222–1409	1,233	ND
pHV1	HVO_C0001 ^d (ori-pHV1) ^a	<i>orc10</i> ^d	101–1327/F 143–1288	1,227	ND
pHV1	HVO_C0057 ^a	<i>orc8</i>	56369–57616/F 56375–57604	1,248	618

^aDeleted.^bEssential.^cPop-in not possible; genome rearrangement occurred.^dPart of origin of replication.^eF, forward; R, reverse.^fND, nondetectable.

In other haloarchaea, only a subset of *orc* genes was found to be essential, e.g., only two of 10 *orc* genes of *Hbt. salinarum* are essential (18). Therefore, it was decided to analyze the essentiality of all 16 *orc* genes of *Hfx. volcanii*. It was attempted to generate in-frame deletion mutants. This approach involves deletion of a major part of the open reading frame but leaves the very 5′-most and the very 3′-most codons intact. It thereby guarantees that the expression of adjacent genes is not influenced. In two cases, the distance to the downstream gene of the same orientation was only 54 nucleotides (nt) (*orc16*) and 60 nt (*orc9*). On the one hand, these distances could include terminators and promoters in the intergenic regions; however, on the other hand, the possibility could not be excluded that these *orc* genes were cotranscribed into bicistronic transcripts. In three cases, the downstream gene had the opposite orientation, and the distances were so small that overlap of the 3′ untranslated regions (UTRs) were suspected, i.e., 47 nt for *orc1*, 18 nt for *orc12*, and −8 nt (= overlap of ORFs) for *orc14*. The exact regions that were chosen for the deletions are summarized in Table 1. The deletion analysis was performed using the so-called pop-in-pop-out method that is well established for *Hfx. volcanii* (19–21). In 12 of the 16 cases, the deletion mutant could be generated successfully. The 12 deletion mutants were analyzed by PCR and by Southern blotting analyses and were shown to be homozygous deletion strains (data not shown). The oligonucleotides used for probe generation, and the sizes of the hybridizing restriction fragments are included in Table S2 in the supplemental material.

The only genes that could not be deleted were *orc11* (HVO_2293), *orc12* (HVO_A0072), and *orc6* (HVO_B0001). In these three cases, the respective pop-in clones could readily be generated, but after the pop-out selection, none of the more than 100 tested clones turned out to be a deletion mutant, but all had retained the wild-type copy of the gene.

Therefore, we concluded that *orc11*, *orc12*, and *orc6* are essential. The phylogenetic analyses did not give any indication why the three genes should be essential. None of the three genes is closely related to the essential genes of *Har. hispanica* and *Hbt. salinarum*. They are also not closely related to one another but are found at different positions in the tree. In addition, *orc11* and *orc6* are in the same clade as nonessential *orc* genes, i.e., *orc14* for *orc11* and *orc7* and *orc8* for *orc6* (compare Fig. 1). Remarkably, *orc6* is the sole *orc* gene of the minor chromosome pHV3, while minor chromosome pHV1 encodes two *orc* genes, and the major chromosome (with the integrated pHV4) contains 13 *orc* genes. Thus, in the future, it could be interesting to analyze whether the integration of further *orc* genes on pHV3 would render *orc6* nonessential.

Taken together, five of the six origin-adjacent *orc* genes were not essential. Therefore, a location adjacent to ORBs or a location far from any origin does not tell anything about the importance of an *orc* gene in *Hfx. volcanii*. The essentiality of one of the 16 *orc* genes, HVO_A0064, could not be analyzed with the same approach as for the other 15 *orc* genes because attempts to generate the pop-in variant failed. Four independent colonies were analyzed by Southern blotting (for oligonucleotides for probe generation, see Table S2 in the supplemental material), and in all cases, neither the wild-type version nor the deletion version of the gene could be detected, indicating that a genomic rearrangement had occurred. In addition, PCR analyses were performed and did not lead to the amplification of the gene, also suggesting that HVO_A0064 was deleted, together with other genes. The gene is flanked by two nearby transposase genes (HVO_A0062 and HVO_A0066); therefore, it seems possible that integration of the deletion plasmid triggered recombination between these genes, leading to the deletion of the intervening genes, including HVO_A0064. Even if a clear-cut analysis via the pop-in-pop-out method was not possible, the viability of the mutant with the genome rearrangement indicated that also HVO_A0064 is not essential.

Table 1 summarizes all *orc* genes of *Hfx. volcanii*; the 12 genes that could be deleted, the three essential *orc* genes, and the gene that could not be characterized are indicated with footnotes. Table 1 also includes the transcript levels deduced from a recent differential transcriptome sequencing (dRNA-Seq) study (22) in which the transcriptomes of exponentially growing cultures under optimal conditions were analyzed. The transcript levels are given as coverage averages because three biological replicates were analyzed, and thus the raw read numbers had to be normalized prior to the calculation of averages. The transcript levels varied widely, from nondetectable (ND) to more than 1,000. In seven cases, the coverage averages were between 40 and 250. For comparison, the coverage averages of selected transcripts are given, as follows: the transcripts for two enzymes of central metabolism, citrate synthase and isocitrate dehydrogenase, had coverage averages of 202 and 262, and the coverage averages of transcripts for the MCM helicase and DNA topoisomerase I were 126 and 167. The inability to detect the transcripts of six *orc* genes might be due to their very low levels. Alternatively, the primary transcripts might be processed, and processed transcripts are not detectable by dRNA-Seq.

Phenotypic analysis of 12 *orc* deletion mutants. The colony morphologies of the 12 deletion strains and the wild type were compared, and no difference could be detected. The same is true for microscopic comparison of the cell morphologies. To unravel whether the absence of a single ORC protein has an influence on the fitness of *Hfx. volcanii* cells, growth experiments were performed. The first approach was to inoculate cultures with exponentially growing precultures and grow them in complex medium under optimal conditions. The results are shown in Fig. 3A (eight deletion mutants) and in Fig. S1A in the supplemental material (four deletion mutants). All 12 deletion mutants grew indistinguishably from the wild type.

Next, cultures were inoculated with 11-day-old stationary-phase precultures. Figure 3B and S1B show that five of the deletion strains had a slight growth advantage compared to the wild type. The growth rates were very similar, so that the main difference was a shorter lag phase of the mutants. One deletion mutant (HVO_1725)

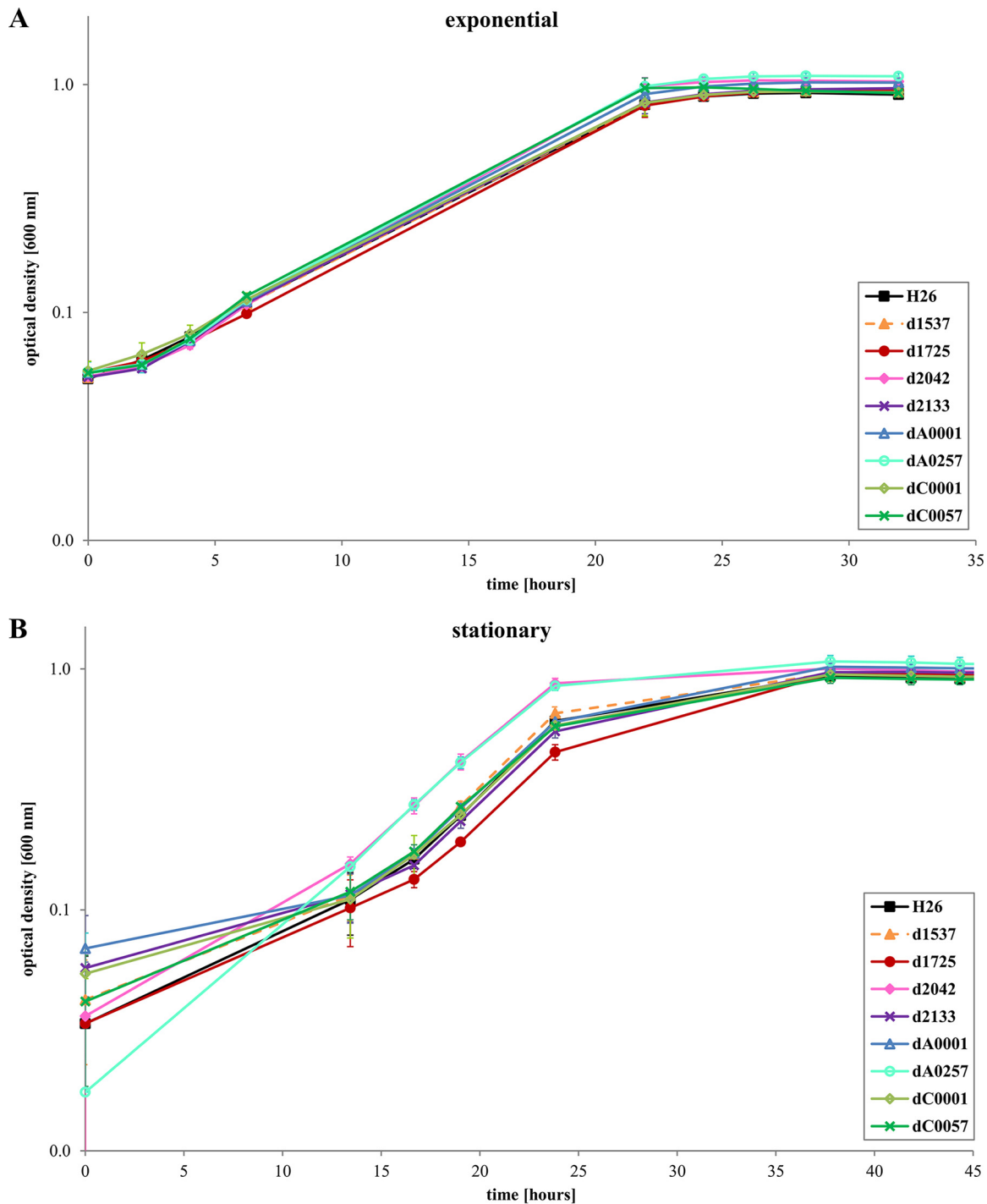


FIG 3 Growth of the wild type and eight *orc* deletion mutants in complex medium. (A) The cultures were inoculated with cells of the mid-exponential growth phase. (B) The cultures were inoculated with stationary-phase cells. Average results from three biological replicates are shown with standard deviations.

had a very slight growth defect, but the difference was very small. The growth yields of all 12 deletion mutants were identical to that of the wild type.

Next, the deletion mutants and the wild type were grown in synthetic medium with glucose as sole carbon and energy source. Figures 4A and S2A show that under this growth condition as well none of the 12 deletion mutants had a growth defect. In contrast, eight deletion mutants had a slight growth advantage. The wild-type and the

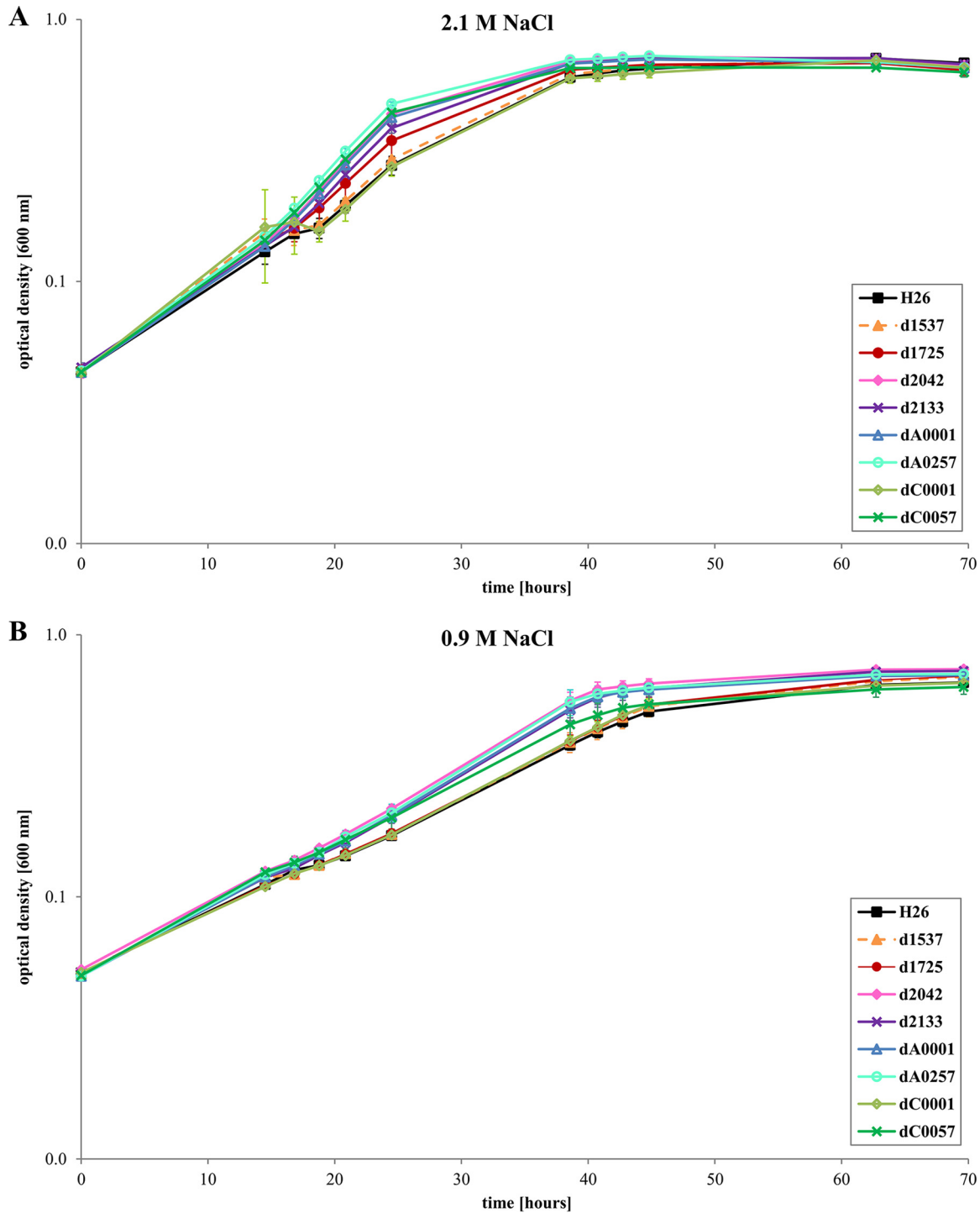


FIG 4 Growth of the wild-type and eight *orc* deletion mutants in synthetic medium with glucose as the sole carbon and energy source. (A) The medium had the optimal NaCl concentration of 2.1 M. (B) The medium had an NaCl concentration of 0.9 M, close to the lowest limit for *Hfx. volcanii*. Average results from three biological replicates are shown with standard deviations.

deletion strains were also grown in glucose synthetic medium with a salt concentration of 0.9 M (Fig. 4B and S2B), which is close to the minimal salt concentration of 0.7 M that *Hfx. volcanii* can survive (23). Again, none of the deletion strains had a growth defect, and four strains grew somewhat faster than the wild type.

In summary, the absence of none of the 12 nonessential ORC proteins resulted in a growth defect; in contrast, several deletion strains exhibited a moderate growth

advantage. We have shown before that single gene deletion mutants of *Hfx. volcanii* can grow better than the wild type. Seven of 27 deletion mutants of regulatory small RNA (sRNA) genes had a growth advantage compared to the wild type under at least one of 10 tested conditions (21). Gain-of-function phenotypes have also been reported for deletion mutants of eukaryotic microRNA (miRNA) genes (24, 25). This indicates that evolution does not select for cells that can grow fastest under one specific (laboratory) condition but for cells that have the most flexible regulatory network, which is able to deal with the manifold and dynamic conditions that a cell can experience in nature.

Quantification of the copy numbers of three chromosomes in the wild-type and in 12 *orc* deletion mutants. To unravel whether the 12 nonessential ORC proteins might be involved in replicon copy number regulation, the copy numbers of the major chromosome (including the integrated pHV4) and the two minor chromosomes pHV1 and pHV3 were determined in exponentially growing cells as well as in stationary-phase cells of the wild type and the 12 deletion mutants. Figure 5 shows that the absence of each of the 12 ORC proteins has an influence on the copy number of at least one of the three replicons. However, the situation is rather complex. Very unexpectedly, the absence of *HVO_0001* led to an increase in the copy number for all three chromosomes under exponential as well as under stationary-phase conditions. The influence of ORC1 on the copy number of the major chromosome and pHV1 is considerable, but the influence on the minor chromosome pHV3 is very high. The absence of *HVO_2292* (ORC14) also led to an increase in the copy number for all three chromosomes; however, in this case, the influence on the major chromosome is very large, in contrast to the rather small influence on the other two chromosomes.

Two other ORC proteins that have an influence on the copy number of all three chromosomes are *HVO_1537* and *HVO_1725* (ORC15 and ORC5). However, the direction of influence is opposite to that in the above-mentioned cases, i.e., their absence resulted in a decrease in copy number. The effect is considerably greater on the major chromosome than on pHV1 and pHV3, and it is restricted to the exponential growth phase. Another regulatory pattern was observed in the *HVO_C0001* deletion mutant. The copy numbers of the major chromosome and pHV1 were decreased in the mutant, whereas, in contrast, the copy number of pHV3 was increased. *HVO_A0257* is an example of an ORC protein that influences only two of the three replicons. Its absence led to a decrease in the copy number of the major chromosome and an increase in the copy number of pHV1 and did not influence the copy number of pHV3. The absence of *HVO_0634* led to an increase in the copy numbers of pHV1 and pHV3 in exponential as well as stationary phases, while the copy number of the major chromosome remained unchanged. Taken together, these results clearly show that a specialization of replication initiation proteins for one specific adjacent replication origin, which has been found to exist for *Sulfolobus* spp. and *Har. hispanica* (8, 11), does not exist in *Hfx. volcanii*. Deletion of all 12 nonessential *orc* genes led to a copy number change of at least two chromosomes in at least one of the two growth phases. In addition, it can be assumed that the three essential ORC proteins also have an influence on the copy number of at least one replicon. Therefore, the copy numbers of the three chromosomes seem to be regulated by a network of many ORC proteins, which can have positive or negative regulatory effects on one or more of the chromosomes. In most cases, the effects were larger in exponential phase than in stationary phase. It has been shown that environmental conditions highly influence the copy number, e.g., the copy number of the major chromosome can be as low as two during phosphate starvation and higher than 40 under phosphate surplus. It is tempting to speculate that individual ORC proteins are specialized in regulating replicon copy numbers in response to specific environmental changes. Further experiments are needed to substantiate this speculation.

Notably, deletion of *HVO_0001* revealed that deletion of an *orc* gene can result in a different phenotype than deletion of the whole replication origin, i.e., the *orc* gene and the adjacent repeat region. While deletion of *HVO_0001* led to an increase in copy number of the major chromosome (Fig. 5), deletion of the whole *oriC1* region led to a

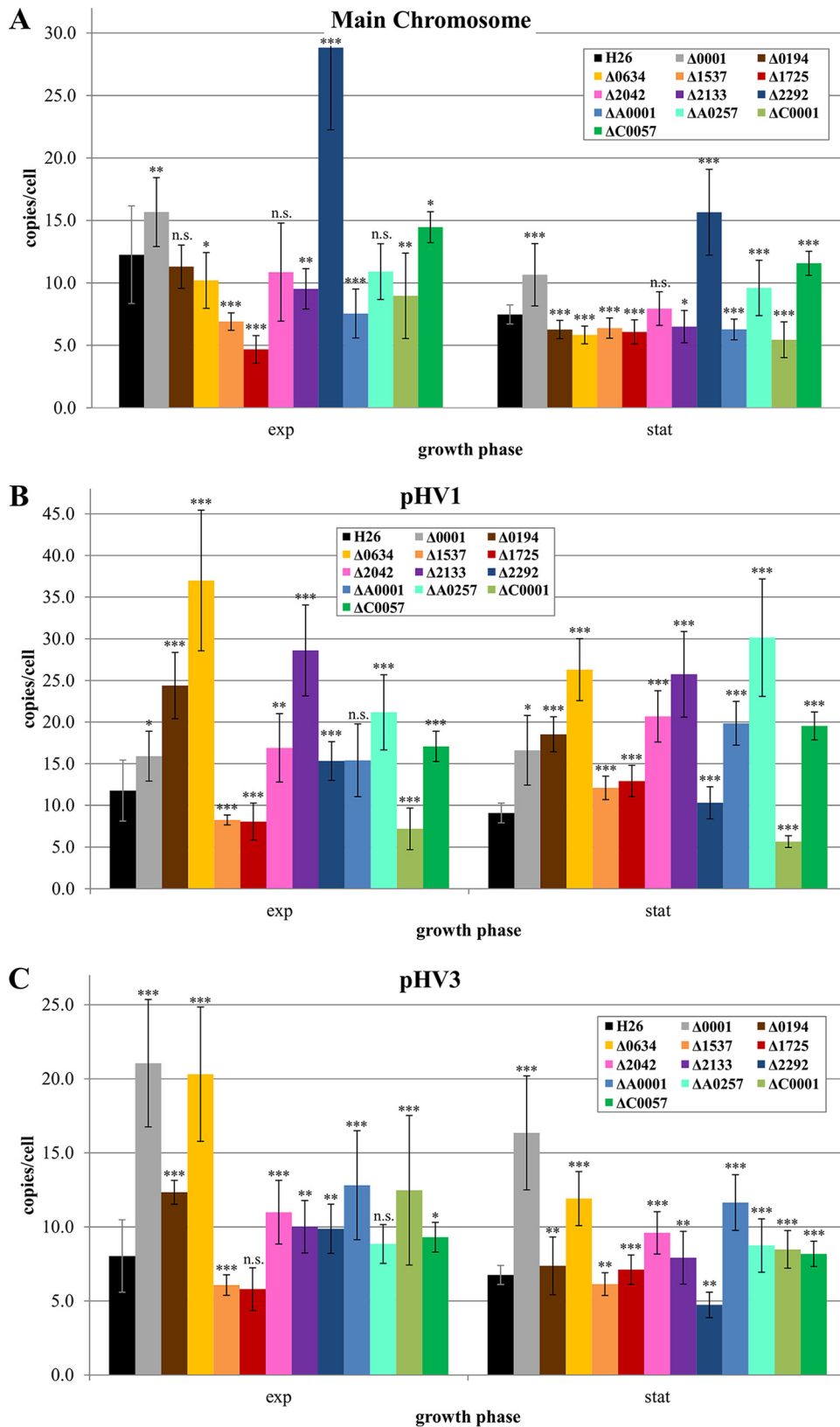


FIG 5 Quantification of the copy numbers of three chromosomes in the wild type and 12 *orc* deletion mutants. The copy numbers were quantified in cells of the early exponential phase (about 1×10^8 to 3×10^8 cells/ml) and in stationary-phase cells using real-time PCR. The results are based on three biological replicates and 24 technical replicates. Significances of differences in copy number were analyzed with Student's *t* test. *, *P* value < 5%; **, *P* value < 1%; ***, *P* value < 0.1%; n.s., not significant.

decrease in copy number (26). The fact that the *oriC1* repeat region without the adjacent *orc* gene has an influence on the chromosome copy number is an additional indication for crosstalk between various ORC proteins and origins. It will be interesting to analyze how many and which ORC proteins can bind to the ORBs of a specific origin.

Conclusions. The evolution of the ORC protein family is very complex, and duplications happened repeatedly at very different points in time. The attempt to delete the 16 *orc* genes of *Hfx. volcanii* revealed that only three of them are essential, while 12 single gene deletion mutants could be generated. None of the 12 deletion mutants had a growth defect, while several deletion mutants grew slightly better than the wild type. The absence of each of the 12 nonessential ORC proteins in the single-gene deletion mutants influenced the copy number of at least one of the three chromosomes. Many ORC proteins influenced the levels of two or all three chromosomes, revealing that the copy number is determined by an intricate regulatory network. Notably, the specialized pairs of autonomous origin repeats with an adjacent *orc* gene, which have been found in other archaeal species, do not exist in *Hfx. volcanii*.

MATERIALS AND METHODS

Construction of phylogenetic trees. Phylogenetic trees were generated using the program MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 (27). Multiple sequence alignments were generated using ClustalW (www.ebi.ac.uk). Low-complexity regions lacking phylogenetic information were removed. Trees were generated using the maximum parsimony and neighbor-joining methods. Bootstrap analyses were performed with 1,000 replications.

Haloarchaeal and bacterial strains. *Haloferax volcanii* H26 was obtained from Thorsten Allers (University of Nottingham, UK) and used as wild-type and parent strain for deletion mutants in this study. H26 is a *pyrE* deletion mutant and is thus auxotrophic for uracil, which can be used as a selection marker. The single-gene deletion strains of 12 *orc* genes were generated in this study.

Escherichia coli XL1 blue MRF was obtained from Stratagene (Amsterdam, The Netherlands).

Media and culture conditions. *Hfx. volcanii* was grown in complex medium (28) and synthetic medium (29) as described. All ingredients were obtained from VWR (Radnor, PA). The complex medium was supplemented with 8 μ M FeSO₄ (P015.1; Roth, Karlsruhe, Germany), 0.1% (vol/vol) SL-6 trace element solution (30) (all from Roth), 1 ml vitamin solution (B6891; Sigma-Aldrich, St. Louis, MO), and 50 μ g/ml uracil (A0667; AppliChem, Darmstadt, Germany). The synthetic medium was supplemented with 10 mM NH₄Cl as N source (21236.267; VWR), 1 mM K₂HPO₄ as P source (6878.2; Roth), 0.5% (wt/vol) glucose as C source (1.08342; Merck, Darmstadt, Germany), and 100 mM morpholinepropanesulfonic acid (MOPS; pH 7.2) (M3183; Sigma-Aldrich). Supplements of the complex medium were also added. For osmotic stress conditions, medium with only 0.9 M NaCl instead of 2.1 M NaCl was used. Cultures were grown in 100-ml Erlenmeyer flasks in a rotary shaker at 42°C and 250 rpm. Phenotypic analyses were performed using growth in microtiter plates (see below).

E. coli XL1 blue MRF was grown under standard conditions (31).

General molecular genetic techniques. General molecular genetic techniques were performed as described by Green and Sambrook (31). Genomic DNA was isolated from *Hfx. volcanii* according to Rosenshine et al. (32). Plasmid DNA was isolated using GenElute plasmid midiprep kit (Sigma-Aldrich), and PCR fragments were either purified directly or from preparative agarose gels using a SeqLab PCR-Combi-kit (SeqLab, Göttingen, Germany). Restriction enzymes and T4 DNA ligase were obtained from Thermo Fisher Scientific (Waltham, MA), and, if not otherwise stated, the enzymes were used according to the instructions of the manufacturer. The *Pfu* and *Taq* polymerases were isolated in house, and oligonucleotides were synthesized by Sigma-Aldrich or biomers.net (Ulm, Germany). *Hfx. volcanii* was transformed as described by Cline et al. (28).

Construction of deletion mutants. The deletion mutants were constructed by the so-called pop-in-pop-out method, as previously described (19, 20). The oligonucleotides used for amplification of the flanking sequences of the respective genes are summarized in Table S1 in the supplemental material. The flanking sequences included a few codons from the beginning and the end of the genes. In each case, the two PCR fragments contained an overlap and could be fused into one fragment by a third PCR. In this way, in-frame deletion variants of the respective *orc* genes were generated. The genomic localizations of the deleted regions are included in Table 1.

The suicide vector pMH101 was digested with EcoRV, and the fused PCR fragments were ligated into the vector using restriction selection cloning. In the cases of $\Delta 2133$ and $\Delta A0257$, the suicide vectors were generated by Gibson assembly (33). The sequences of all plasmids were verified by sequencing (GATC, Constance, Germany). The genomic organizations of deletion mutants were verified by Southern blotting analyses as described below.

Southern blot analysis for verification of deletion mutants. Southern blot hybridization was performed according to Lange et al. (34). The oligonucleotides, as well as the restriction enzymes used and the expected fragment sizes, are summarized in Table S2.

Phenotypic analysis of ORC deletion mutants. Phenotypic characterization was generally performed as described by Jantzer et al. (23). Cultures in complex medium were either inoculated with exponentially growing precultures or with cultures that had been in stationary phase for 11 days.

Cultures in synthetic medium were inoculated with exponentially growing precultures in the same medium. The cultures were harvested by centrifugation at 8,000 rpm for 5 min, the supernatant was discarded, and the pellet was washed with basal salt solution (medium without a C, N, or P source). After resuspension, the cells were used to inoculate new culture to an optical density at 600 nm (OD_{600}) of 0.05. Only the inner 60 wells of microtiter plates were used for growth; the outmost wells were filled with 200 μ l of 1 M NaCl as an evaporation barrier. Growth was monitored with a microtiter plate spectrophotometer. Three biological replicates were performed and average values and standard deviations were calculated.

Quantification chromosome copy numbers using quantitative real-time PCR (qPCR). For quantification of chromosome copy numbers, a previously established real-time PCR technique was used (16, 17, 34). As a standard fragment for genome copy number determination, a 1-kbp fragment of *leuB* was amplified by PCR. For pHV1 and pHV3 copy number determination, respectively, a 1-kbp fragment of *HVO_C0001* and a 1-kbp fragment of *HVO_B0001* were amplified. In the case of $\Delta C0001$, a 1-kbp standard fragment downstream of *HVO_C0001* was generated and two different pairs of qPCR oligonucleotides were used to ensure reproducibility of the determined copy number. The standard fragments were purified from a preparative agarose gel as described above and precipitated with ethanol. They were dissolved in Tris-EDTA (TE) buffer, and the DNA concentration was determined by photometric measuring with a NanoDrop ND-1000 spectrophotometer. The concentration of the standards was adjusted to 100 to 200 ng/ μ l. All oligonucleotides used for standard fragment PCR and real-time PCR are described in Table S3 in the supplemental material.

For determination of chromosome copy numbers, 1×10^8 cells of early exponential (1×10^8 to 3×10^8 cells/ml) and early stationary-phase cultures (1×10^9 to 3×10^9 cells/ml) in complex medium were harvested by centrifugation at 13,000 rpm for 5 min. The supernatant was discarded and the pellet was resuspended in 100 μ l of basal salt solution and lysed by addition of 900 μ l of distilled water. An aliquot of 40 μ l of cell extract was dialyzed for 3 h on membrane filters (Millipore, 13 mm diameter, VSWP01300; Merck) against distilled water. One qPCR mixture contained 0.8 μ M of each oligonucleotide, 5.5 μ l nuclease-free water, 12.5 μ l Maxima SYBR green qPCR master mix (K0223; Thermo Fisher Scientific), and 5 μ l of standard or cell extract, to a total volume of 25 μ l. Serial dilutions (10^{-3} to 10^{-8}) of the standard fragment in duplicate and four different dilutions of three biological replicates, also in duplicate, were used for qPCR, so that in total 24 technical replicates were used for copy number determination. A negative control with omission of template DNA was also performed. qPCR conditions were as follows: 10 min at 96°C, 50 cycles with 30 s at 96°C, 30 s at 64°C, and 30 s at 72°C, followed by 5 min at 72°C and a melt curve analysis from 62 to 96°C in 1°C steps.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00161-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.8 MB.

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