

Localizing genes on the map of the genome of *Haloferax volcanii*, one of the Archaea

(archaea/halobacteria/genome/mapping)

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Communicated by Carl R. Woese, November 25, 1991

ABSTRACT We have assigned genetic markers to locations on the physical map of the genome of the archaeon *Haloferax volcanii*, using both a physical method (hybridization) and a more specific genetic technique (transformation with cosmids). Hybridizations were against restriction digests of each of 151 cosmids making up a minimally overlapping set and covering 96% of the genome. Results with a cloned insertion sequence and a tRNA probe indicated that transposable elements are concentrated on two of the four plasmids of this species, whereas regions complementary to tRNA are largely chromosomal. For a genetic analysis of genes involved in the biosynthesis of amino acids, purines, and pyrimidines, we used cosmid transformation to assign 139 of 243 ethyl methanesulfonate-induced auxotrophic mutations, generated and characterized for this study, to single cosmids or pairs of cosmids from the minimal set. Mutations affecting the biosynthesis of uracil, adenine, guanine, and 14 amino acids have been mapped in this way. All mutations mapped to the 2920-kilobase-pair chromosome of *Hf. volcanii* and seemed uniformly distributed around this circular replicon. In some cases, many mutations affecting a single pathway map to the same or overlapping cosmids, as would be expected were genes for the pathway linked. For other biosynthetic pathways, several unlinked genetic loci can be identified.

In 1977, Woese and Fox (1) proposed that methanogens, the extreme halophiles, and some thermophilic bacteria comprise a prokaryotic kingdom, the archaeobacteria, as different from the better-known eubacteria as these latter were from the eukaryotes. Research in molecular biology and biochemistry in the 14 yr since has fully supported this contention (2, 3) and provided, most recently, the basis for a rooted universal phylogenetic tree (4). In this tree, archaeobacteria and eukaryotes (more precisely the nuclear–cytoplasmic lineage of eukaryotes) appear as sister groups, and eubacteria diverge at the base of the tree. In part because of this rooting, Woese *et al.* (2) have renamed the archaeobacteria Archaea, emphasizing their distinctness from the rest of the prokaryotes.

Archaeal gene and genome structure and function have until recently been investigated by indirect methods—comparing the coding sequences of genes that can be cloned with easily available probes with the coding sequences of their eubacterial/eukaryotic homologs or looking for constant features in sequences flanking nonhomologous archaeobacterial genes. To make possible more sophisticated and detailed genetic analyses, we developed techniques for transformation of the halophilic archaeon *Haloferax volcanii* with phage, plasmid, and chromosomal DNA (5–7) and con-

structed a shuttle vector that can be selected and maintained in either *Hf. volcanii* or *Escherichia coli* (8).

We also used libraries of *Hf. volcanii* DNA in a cosmid vector (Lorist M) to construct a bottom-up physical genome map. We characterized [landmarked (9)] over 2000 of the clones by single and double restriction enzyme digestion and linked 151 of them (the minimal set) to produce a detailed restriction map (903 sites, ref. 10). We found that the genome consists of a large 2920-kilobase-pair (kbp) circle, which we call the chromosome, and circles of 690, 442, 86, and 6.4 kbp, designated, respectively, as plasmids pHV4, pHV3, pHV1, and pHV2. Restriction sites for the six mapping enzymes were unevenly distributed in the genome, being 5-fold more frequent in two chromosomal and three plasmid regions we called oases than elsewhere. We suggested that these oases comprise part of the relatively A+T-rich DNA fraction detectable in this species (10). In another halobacterium, *Halobacterium halobium*, the A+T-rich fraction (FII) selectively harbors insertion sequence elements (11, 12).

A physical map gains interest and utility from its conversion to a genetic map, and here we report results obtained by two quite different methods for effecting such conversion. (i) Hybridization with bulk or cloned probes has localized for us some 45–50 putative or confirmed tRNA-encoding regions and a like number of copies of insertion sequences related to the previously described insertion sequence element ISH51, giving a general picture of the distribution of both phenotypically essential and probably dispensible genetic elements within and between chromosome and plasmids. (ii) The transformation to prototrophy of auxotrophic mutants isolated for this study with cosmids from the minimal set has allowed us to assign precise map positions to 139 mutations affecting the biosynthesis of uracil, adenine, guanine, and 14 amino acids, providing information on the distribution of protein-coding genes and a starting point for detailed analyses of linkage and coordinated activity in several specific gene systems. Together, these results encourage us to draw some general conclusions about genetic organization in this archaeon.

MATERIALS AND METHODS

Materials, Bacteria, and Culture Methods. Enzymes were purchased from Boehringer Mannheim and Pharmacia. Ethyl methanesulfonate, methyl methanesulfonate, amino acids, and vitamins were from Sigma. *Hf. volcanii* WFD11 (7) is a derivative of DS2 cured of the endogenous plasmid pHV2 and was the parent of all mutants described here, except for the ornithine auxotroph (see Table 1), which was a gift from M. Mevarech (Department of Microbiology, Tel Aviv University). Cultures were grown with shaking at 37°C on rich or

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Abbreviation: ISH, halobacterial insertion sequence.
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minimal medium (8, 13). Rich medium consists of a medium salt solution (3.5 M NaCl/0.15 M MgSO₄/0.05 M KCl/7 mM CaCl₂/1 mM MnCl₂/50 mM Tris·HCl, pH 7.2) with 3 g of yeast extract (Difco) per liter and 5 g of enzymatic hydrolysate of casein (Sigma) per liter. Minimal medium contains this same salt solution, supplemented with 0.2 μM CuSO₄/12 μM FeSO₄/1.8 mM MnCl₂/1.5 μM ZnSO₄/10 mM NH₄Cl/0.1 mM K₂HPO₄, pH 7.0/0.1% sodium succinate (wt/vol)/1% (vol/vol) glycerol. For plates, Difco Bacto agar was added to 2%, and incubation was at 42°C. When required, amino acids were supplied at 50 μg/ml, purines and pyrimidines at 25 μg/ml, and vitamins at 0.25 μg/ml. Cosmids were maintained in *E. coli* strain ED8767 and purified as described (10).

Mapping by Hybridization. Probes for repeated sequences and tRNAs were hybridized to dot-blot of cosmid preparations and, for more precise localization within cosmids, to Southern transfers of *Mlu* I-digested cosmid DNAs. The dot-blot was produced by applying the cosmid DNAs (≈200 ng) to pieces of Whatman 3MM paper bonded to glass by pressing them onto a layer of Parafilm M. Multiple impressions of this DNA-impregnated stencil could then be made by applying a prewetted piece of GeneScreen (DuPont/NEN) membrane backed by a layer of Whatman 3MM paper soaked with 0.4 M NaOH and rolling with an 18-mm culture tube. A dozen identically arranged filters bearing similar amounts of DNA could be produced with a single pipetting.

Southern transfers of cosmid DNAs were made by the alkaline transfer method and hybridized as described (9). The probes for ISH51 were the gel-purified 716-base-pair (bp) *Eco*RI (left) and 736-bp *Eco*RI-*Xho* I (right) fragments of p7X4.1 (14). The D element probe was the 10.2-kbp *Mlu* I fragment of pHV1. The cloned tRNAs were pVT1, -2, -6, -9, -29, and -38 of Daniels *et al.* (15) and pHvE420-5, pHvE308, and pHvH202-5L2 [tRNA^{thr} (GGU), tRNA^{gly} (CCC), and elongator tRNA^{met} (AUG), ref. 16 and R. Gupta, personal communication]. Total tRNA probe was 3'-end-labeled after salt and 2-propanol precipitation of other nucleic acids, and gel purified as described (9). The preparation also contains 5S and 7S RNAs (whose gene locations are known), but extensive work by Gupta and colleagues (16, 17) shows that all of the detectably labeled species in such preparations are tRNAs.

Mutagenesis and Mutant Selection. WFD11 was grown in rich medium to midlogarithmic phase. Cells were harvested by centrifugation and resuspended in medium salts containing 0.2 M Tris·HCl, pH 7.5. Ethyl methanesulfonate (at 11 μl/ml for 120 min) or methyl methanesulfonate (at 2 μl/ml for 40 min) were used at 37°C with shaking. Mutagenized cells were diluted with 50 vol of medium salts and washed with medium salts before suspension in 10 vol of rich medium. Cells were then allowed to recover and grow overnight, with shaking at 37°C, before plating on rich medium (13).

Plates were incubated 10–14 days, until colonies appeared. Colonies were replicated with velvet onto rich and minimal medium and reincubated. Putative auxotrophs were picked and streaked on medium containing pooled supplements, as described by Davis *et al.* (18).

Transformation of *Hf. volcanii*. Freshly prepared or previously frozen suspensions of auxotrophic cells were treated with EDTA and transformed with DNA samples dissolved in 100 mM EDTA, pH 8, according to a procedure that we detailed before (6, 19) in microtiter wells at room temperature. In addition to transformations with individual or pooled cosmids (see *Results* and *Discussion*), each auxotrophic mutant was also transformed with wild-type DNA (as a positive control) and with a sample containing no DNA (as a mock transformation).

Map Assembly. We have elsewhere described an ordered set of 151 cosmids (plus the plasmid pHV2) that covers all but 4% of the *Hf. volcanii* genome (10). Based on restriction

analyses of these cosmids (and many others, some of which we included in our transformation experiments) and on Southern hybridization patterns to restriction digests of entire *Hf. volcanii* DNA, a restriction map of the *Hf. volcanii* chromosome has been assembled (10). Most of the cosmids identified as bearing genes by hybridization or transformation were positioned by referring directly to this physical map. Cosmids of interest that did not belong to the final minimal cosmid set were localized by aligning their individual restriction maps to the completed global map.

RESULTS

Localizing tRNA and Insertion Sequence Elements by Hybridization. For an overview of the distribution of essential genes (those for tRNA) and dispensable and possibly parasitic genetic elements [insertion sequence ISH51 (14)] we hybridized Southern blots of *Mlu* I digests of the minimal set of cosmids with four types of probe: (i) available cloned tRNA genes (refs. 15 and 16; R. Gupta, personal communication) specific for valine (GAC), tryptophan (CCA), lysine (CUU), serine (GCA), cysteine (CGA), threonine (GGU), glycine (CCC), and methionine (both initiator and elongator species); (ii) an end-labeled bulk tRNA preparation; (iii) internal probes from ISH51; and (iv) another element related to ISH51, which we designate repeat D, found after using *Mlu* I fragments from plasmid pHV1 as probes against total genomic blots. Because copies of ISH51 often contain an *Mlu* I site, multiple closely spaced copies of the element can be enumerated by Southern hybridization. For tRNA-positive fragments, we do not know whether one or more tRNA-hybridizing sequences are present on a given *Mlu* I fragment.

Known tRNA genes and sequences hybridizing to the labeled total tRNA preparations are almost exclusively confined to the 2920-kbp chromosome and seem uniformly scattered around this circle (Fig. 1A). ISH elements, on the other hand, are concentrated in plasmids pHV1 and pHV4 (60% of the total elements, Fig. 1B). Plasmid pHV1 is one large restriction site oasis (region where sites for restriction enzymes used in mapping are unexpectedly frequent), whereas on pHV4, ISH51 elements are concentrated in two such oases (indicated by thick black lines in Fig. 1B). Malachite-green bisacrylamide column chromatography (performed as in ref. 14, data not shown) of sixteen *Mlu* I fragments of pHV1 confirm that all are relatively A+T-rich FII DNA, whereas cosmids from chromosomal regions lacking oases are more G+C-rich (FI DNA). Thus, the FII fraction is enriched in ISH51 elements, although they are by no means restricted to those FII regions that we can identify as oases by restriction site mapping. On the chromosome, ISH51 elements show some preference for the region from 2600 to 2900 kbp, where one of the two small chromosomal oases is also found.

Isolation and Identification of Mutants. To develop more properly genetic approaches to mapping the *Hf. volcanii* genome, we needed an extensive collection of auxotrophic mutants. Mevarech and Werczberger (13) had described 23 ethyl methanesulfonate-induced *Hf. volcanii* mutants requiring adenine, guanine, or one of seven amino acids. We used these methods and ethyl methanesulfonate or methyl methanesulfonate to obtain a collection of 428 mutants that produce colonies on rich (yeast extract- and enzymatic casein hydrolysate-containing) medium but do not produce replicas on minimal agar plates.

Eleven supplement pools, each containing five supplements, were constructed as described by Davis *et al.* (18). Supplements are typically present in two different pools, and patterns of growth of each of the 428 mutant strains on plates spread with pools were scored for preliminary identification of amino acid (all 20), purine (adenosine or guanosine),

Table 1. Auxotrophic mutations mapped to cosmids

| Nutritional requirement (symbol)* | Number of mutants [†] | Number mapped | Cosmid(s) identified |
|---|--------------------------------|---------------|----------------------|
| Adenine (<i>ade</i>) | 29 (1) | 1 | 2D7 |
| Arginine (<i>arg</i>) | 19 (19) | 4 | 21 |
| | | 14 | 21, 247 [‡] |
| Glutamine (<i>gln</i>) | 3 (2) | 2 | 460 |
| Guanosine (<i>gua</i>) | 44 (17) | 13 | 5G7 |
| | | 2 | 347 |
| | | 1 | 497 |
| Histidine (<i>his</i>) | 26 (23) | 6 | 499 |
| | | 1 | G171 [‡] |
| | | 6 | G171, G411 |
| | | 1 | G171, 32 |
| | | 2 | H10 |
| | | 2 | 347 |
| Leucine (<i>leu</i>) [§] | 24 (21) | 16 | 56 |
| Lysine (<i>lys</i>) | 16 (16) | 14 | 2D7 |
| Methionine (<i>met</i>) | 4 (4) | 2 | 501 |
| | | 1 | 118, A176 |
| Ornithine (<i>orn</i>) [¶] | 1 (1) | 1 | 21 |
| Phenylalanine + tyrosine (<i>pty</i>) | 17 (9) | 1 | G411 |
| | | 1 | 410 |
| | | 1 | 501 |
| Phenylalanine + tyrosine + tryptophan (<i>aro</i>) | 2 (2) | 1 | G411 |
| Serine (<i>ser</i>) | 1 (1) | 1 | 499 |
| Threonine (<i>thr</i>) | 5 (5) | 4 | 126 |
| Tryptophan (<i>trp</i>) | 29 (29) | 19 | G203** |
| | | 10 | 452 |
| Uracil (<i>ura</i>) | 5 (5) | 2 | 266 |
| | | 1 | 478 |
| | | 1 | 166 |
| Uracil + arginine (<i>pyr</i>) ^{††} | 6 (6) | 1 | 128 |
| | | 2 | 128, 208 |
| | | 1 | 478 |
| | | 1 | 531 |
| Valine + isoleucine (<i>ilv</i>) | 12 (4) | 3 | 501 |

*Specific gene names have not been given, except for the sequenced tryptophan genes (25), and we make no claims for homology to similarly named genes in *E. coli*.

[†]Of the 243 auxotrophic mutants listed, 165 were used in transformation experiments. The number of mutants of each class used is given in parentheses. Where the total number successfully mapped is less than this, high reversion rates precluded assignment to cosmids, or no cosmids tested gave transformants (eight instances).

[‡]Because some mutants are in the region overlapped by two cosmids, whereas others can be transformed by only one cosmid, we assume that several genes involved in this pathway are located in this part of the chromosome.

[§]Growth studies show at least two classes of leucine auxotroph (response to α -ketoisocaproate).

[¶]This mutant was provided by Moshe Mevarech.

^{||}Mutants requiring both phenylalanine and tyrosine, but not tryptophan, should lie beyond chorismate in the aromatic pathway. Eubacteria show a variety of bifunctional and phenylalanine- or tyrosine-specific enzymes for subsequent steps (32).

**This cosmid replaces cosmids A159 and 488 described previously (10).

^{††}In eubacteria, mutants requiring arginine and a pyrimidine usually map to one locus (*pyrA* in *Salmonella typhimurium*, *carAB* in *E. coli*) (33).

Of the 165 identified auxotrophic mutations transformed, 139 could be assigned to cosmids in this way (Fig. 1A). These include mutations resulting in requirements for adenine,

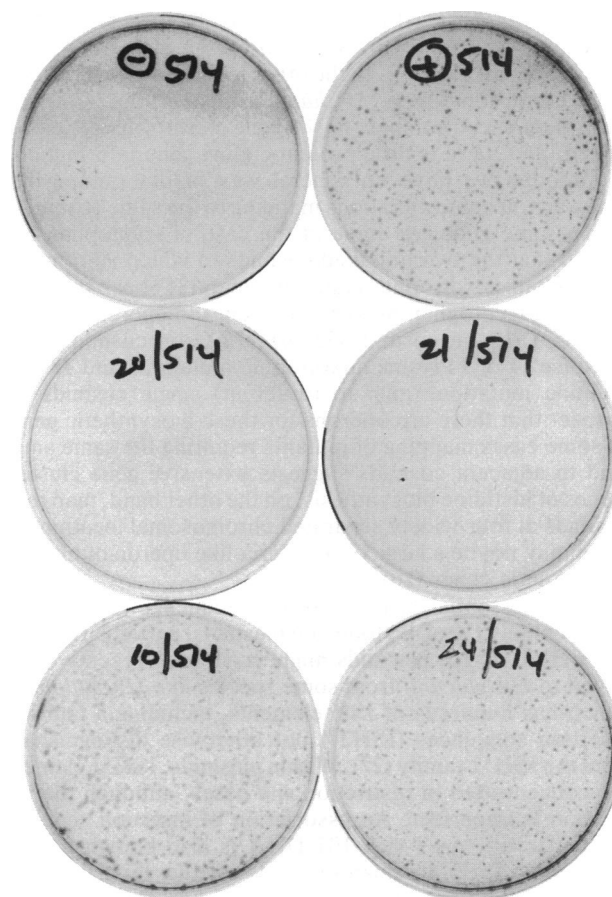


FIG. 2. Transformation of a leucine auxotroph. Of the 25 cosmid pools used, pool 10 and pool 24 transformed WFD514 to prototrophy. The rest of the cosmid pools (for example, pool 20 and pool 21) did not. Mock transformation with no DNA (514/-) produced no colonies on minimal agar plates, whereas total wild-type DNA (514/+) efficiently transformed the leucine auxotroph.

guanosine, uracil, and ornithine and mutations of at least 20 loci resulting in requirements for 1 or more of 14 of the standard 20 amino acids. The remaining 26 mutants either exhibited high levels of reversion (18 mutants) or appeared not to correspond to the 96% of the genome cloned in our cosmid set (eight mutants). In all cases where a single mutant could be transformed to prototrophy with more than a single cosmid, physical mapping methods independently show that the transforming cosmids overlap. Although cosmids representing the entirety of the four endogenous plasmids identified in this genome were included in all transformation tests, all of the 140 mutations mapped proved to be chromosomal. Auxotrophic markers seemed uniformly scattered around the chromosome. The region from 2600 to 2900 kbp (Fig. 1A), where ISH elements are frequent, showed none, but several sequenced and presumably essential tRNA genes do occur in this region, and at least one protein-coding gene can be assigned to this region by hybridization (10).

DISCUSSION

These results from physical and genetic localization of genes provide a preliminary picture of genome organization in an archaeon. The largest circular component of the genome carries all of the at least 35 different biosynthetic enzyme genes mapped and all but three of the 46 regions hybridizing with the bulk tRNA probe. Results obtained with this probe together with those from cloned tRNA genes show no striking clustering of genes for tRNA [as there is, for instance, in

Bacillus subtilis (21)] or of genes involved in biosynthetic functions [as there is in *Streptomyces griseus* or *Pseudomonas* species (22, 23)]. In the other archaeon for which there is a chromosome map [*Methanococcus voltae* (24)], previously sequenced complex tRNA gene clusters appear closely linked, and there is little protein-coding gene information.

Although we have no general way of determining how many auxotrophic mutations mapping to the same cosmid are in the same gene, we have in the case of tryptophan confirmed that the two unlinked cosmids to which all of our 29 tryptophan auxotrophic mutations map (25) bear the seven expected tryptophan biosynthetic genes in operon-like clusters (*trpCBA* on cosmid 452, *trpDFEG* on cosmid G203). Because 14 of 14 lysine auxotrophic mutations and 18 of 18 arginine mutations map to (different) single cosmids, we suspect that there are operons for these biosynthetic genes. In some cases mapping of mutants requiring the same amino acid to adjacent cosmids suggests extensive gene clusters. Genes of histidine biosynthesis, on the other hand, map to six cosmids at four widely separated chromosomal locations, so there may not be a large (eu)bacteria-like operon of histidine biosynthetic genes (26).

Unlike essential genes, insertion sequence elements are preferentially (30 of 49) found on plasmids, particularly pHV4 and pHV1. These plasmids might have been recently introduced to *Hf. volcanii* from some species like *Hb. halobium*, which has hundreds of ISH elements, including a family of insertion sequences (ISH27) not otherwise distinguishable from the ISH51 family (27). Within plasmids, ISH51 elements are concentrated in restriction site oases, although the correlation is imperfect. An association of insertion sequence elements with A+T-rich FII DNA is also known for *Hb. halobium* (11, 12). It remains unclear whether this association reflects a simple preference for A+T-rich targets by ISH elements or results from the confinement of these elements (themselves relatively A+T-rich) to plasmids and specific chromosomal regions for other reasons, such as selection against disruption of essential genes.

Comparisons between archaea, bacteria (eubacteria), and eukaryotes can be made at several levels of genetic and biological organization. Using ancient duplications to root the universal tree, Iwabe and coworkers (4) have provided strong evidence that archaea and eukaryotes are, in fact, the most recently diverged of the three kingdoms, and the sequences of components of the transcription and translation apparatus are consistent with the notion that archaea and eukarya are sister groups (28, 29). In terms of gene organization and clustering, however, archaea look very much like bacteria. Not only are there many examples of operon-like clustering (and cotranscription) of genes for related functions, but there are several cases of large archaeal clusters so similar in gene order to bacterial clusters [RNA polymerase genes, ribosomal protein, and elongation factor genes (28, 29)] that we can only conclude that such specifically ordered clusters were present in the last common ancestor of all cells and faithfully maintained in compact prokaryotic genomes. At the level of overall genome organization, we can as yet say little. At first glance, the genetic map of *Hf. volcanii* may look like a "typical" eubacterial genome map, but we have only the simplest notions about conserved features of bacterial genomes and the roles of functional constraint and history in explaining them. With additional physical and genetic mapping techniques, the necessary comparative data base should build quickly. Especially interesting will be data on the organization of early-diverging eukaryotic genomes of comparable size [such as the 12-megabase-pair chromosome of *Giardia* (30) and the, perhaps even smaller, genome of the microsporidian *Nosema* (31)].

We thank Steve Cline for help in the transformation experiments and the Medical Research Council and Natural Sciences and Engineering Research Council of Canada for support. W.F.D. is a Fellow of the Canadian Institute for Advanced Research.

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