AMBER SUPPRESSION IN THE ARCHAEBACTERIUM *HALOFERAX VOLCANII*

by

Josephine Yau

Hon. B.Sc., The University of Waterloo, 1991.

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTERS OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

Department of Biochemistry and Molecular Biology

We accept this thesis as conforming

to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

February 1994

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

(Signature)

Department of BIOCHEMISTRY AND MOLECULAR BIOLOGY

The University of British Columbia
Vancouver, Canada

Date 17 FEB 94

DE-6 (2/88)

ABSTRACT

The purpose of this project was to test whether amber suppression can occur in *Haloferax volcanii* or not, and if so, to construct a *H. volcanii* strain that can suppress amber mutations. To achieve this goal, a putative amber suppressor was constructed from the tyrosine transfer RNA of *H. volcanii*. Its ability to recognize an amber stop codon and to restore the wild type function of an amber mutated gene was tested.

The gene coding for tyrosine tRNA of *H. volcanii* DS2 was cloned and sequenced. Site-directed mutagenesis was carried out to change the anticodon of the tRNA gene from GUA to CUA, which recognizes the tyrosine codon UAU and UAC, and the amber stop codon UAG respectively.

The *hisC* gene of wild type *H. volcanii* was obtained and recloned into pGEM7(-). Site directed mutagenesis was carried out to change the DNA sequence of its first tyrosine codon (TAC) to an amber stop codon (TAG).

I attempted to replace the wild type *hisC* gene in the *H. volcanii* WFD11 genome with the *hisC* gene carrying the amber mutation. However, although the construct carrying the *hisC*(Am) gene and an antibiotic resistance marker integrated into the genome at the correct place, displacement of the wild type gene through reverse recombination did not occur.

An attempt to test amber suppression in *H. volcanii* was carried out. The *hisC*(Am) gene was introduced into the genome of a mutant strain of *H. volcanii* WR256 (*his*$^-$, *arg*$^-$) with the antibiotic selection marker mevinolin. The

transformants were then transformed again with a plasmid that carries the putative amber suppressor (the $tRNA^{Tyr}$ gene with the mutated amber anticodon) and the other antibiotic selection marker novobiocin. Transformants were then selected with both antibiotics and then tested for restoration of histidine auxotrophy. All transformants still required histidine for growth. Southern hybridization showed that the *hisC*(Am) gene was not integrated into the genome. Mevinolin resistance in the transformants was due to a double crossover recombination event of the antibiotic resistance gene into the genome to replace the wild type gene. Therefore I was not able to conclude whether amber suppression can occur in *H.volcanii* or not.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

A	adenosine
ATP	adenosine triphosphate
bop	bacteriorhodopsin
bp	base pair
^0C	degrees centigrade (Celcius)
C	cytosine
CsCl	cesium chloride
D	dihydroxyuridine
dH_2O	distilled, sterile water
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleoside triphosphate
EDTA	ethylene diamine tetraacetic acid
G	guanosine
his	histidine
Hvo	*Haloferax volcanii*
kbp	kilobase
M	molar
mev	mevinolin
mev^r	mevinolin resistant
µl	microlitre

ml	millilitre
mRNA	messenger ribonucleic acid
nov	novobiocin
nov^r	novobiocin resistant
O.D.	optical density
oligo	oligonucleotide
p	plasmid
PEG	polyethylene glycol
PNK	polynucleotide kinase
pmol	picomole
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal RNA
S	Svedberg unit of sedimentation coefficient
SDS	sodium dodecyl sulphate
ss	single-stranded
su	suppressor
T	thymidine
TE	10mM Tris-Cl, 1mM EDTA
tyr	tyrosine
tRNA	transfer ribonucleic acid
U	uridine

u	unit
ura	uracil
Ψ	pseudouridine
YT	8g/l Bacto trytone, 5g/l Bacto yeast extract, 5g/l NaCl

ACKNOWLEDGMENTS

I thank the members of my supervisory committee, Dr. R. T. A. McGillivray and Dr. P. Candido, for their help and advice during the course of my studies. I also thank my supervisor, Dr. P. P. Dennis, for his advice and the opportunity to work in his lab.

I thank the members of the lab (Luc Bissonette, Janet Chow, Peter Durovic, Phalgun Joshi, Daiqing Liao, Shanthini Mylvaganam, Simon Potter and Janet Yee) for their support and advice. I also thank Deidre de Jong-Wong for her technical help. Special thanks to Mark M. K. Lee for helping me with the computer scanning. I also thank the M. Smith lab, especially Chi-Yip Ho for advice and help with site-directed mutagenesis. Last, but not least, I would like to thank Dr. R. Redfield for her help and advice.

I would also like to acknowledge the financial support of the Natural Science and Engineering Research Council.

DEDICATION

This thesis is dedicated to my parents, Paul and Molly Yau, and to Eric Leung, for their support and encouragement.

INTRODUCTION

1.1 PURPOSE OF THE PROJECT

The goal of this project was to study amber suppression in the archaebacterium *H. volcanii*. Amber suppression has been widely used in *E. coli* for studying gene function and expression by providing a system to isolate conditional mutants in an essential gene. To understand amber suppression, some general background on the genetic code, transcription and translation, transfer RNA properties and functions, and suppression of mutations will be discussed in the following sections.

Haloferax volcanii is a member of the archaebacteria, a group only recently recognized as a separate line of evolutionary descent from a common primordial ancestor, apart from the eubacteria and eukaryotes (Woose *et al.*, 1990). The study of archaebacteria helps us to better understand the universal ancestor and the evolution of cells. Although recent advances in the development of archaebacterial genetics have led to a better understanding of the unique phenotypic and genetic characteristics of the group, our knowledge is still very limited. The development of an amber suppressing archaebacterial strain would be useful for the study of archaebacterial gene functions and the manipulation of genetic material in the future.

1.2 THE GENETIC CODE

The genetic code (Table 1) uses the nucleotide sequence of genomic DNA to specify the order of amino acids in protein produced by a cell or organism (Crick, 1966). The vast array of different proteins possess both structural and catalytic properties that determine the cell's unique abilities, relating to metabolism, growth

	U	C	A	G	
U	UUU Phe UUC Phe UUA Leu UUG Leu	UCU Ser UCC Ser UCA Ser UCG Ser	UAU Tyr UAC Tyr UAA* *Stop* UAG* *Stop*	UGU Cys UGC Cys UGA* *Stop* UGG Trp	U C A G
C	CUU Leu CUC Leu CUA Leu CUG Leu	CCU Pro CCC Pro CCA Pro CCG Pro	CAU His CAC His CAA Gln CAG Gln	CGU Arg CGC Arg CGA Arg CGG Arg	U C A G
A	AUU Ile AUC Ile AUA Ile AUG† Met	ACU Thr ACC Thr ACA Thr ACG Thr	AAU Asn AAC Asn AAA Lys AAG Lys	AGU Ser AGC Ser AGA Arg AGG Arg	U C A G
G	GUU Val GUC Val GUA Val GUG† Val	GCU Ala GCC Ala GCA Ala GCG Ala	GAU Asp GAC Asp GAA Glu GAG Glu	GGU Gly GGC Gly GGA Gly GGG Gly	U C A G

TABLE 1: The Genetic Code

Taken from Watson *et al* (1987), p.437

and division. The characteristic features used to encode protein sequence information were first elucidated for the organism *E. coli* and are now recognized to be nearly universal and therefore generally applicable to all other organisms.

The code consists of triplets; three successive nucleotide base pairs in DNA specify one of the twenty different amino acids found in protein. Triplet codons are non-overlapping, read sequentially in a 5′ to 3′ direction, and when read in sequence specify the order of amino acids from the N (amino) to the C (carboxyl) terminus of the protein. The genetic code is degenerate; using the standard bases found in DNA and RNA, there are sixty-four different triplet combinations, but there are only twenty amino acids found in protein. Examination of the codon assignments indicates that the third base in the triplet is least important and often carries little or no information. They are given the name wobble bases. For example, the amino acid glycine is specified by four codons which differ only in the third position: GGG, GGC, GGA, and GGU. These properties help to minimize the deleterious effects of mutations that occur at the third codon position and simplify the decoding process.

The genetic information carried in DNA is not decoded directly. Instead the sequence from the coding strand of the DNA is first transcribed onto messenger RNA. The mRNA is then decoded on the ribosomes by a series of interactions with amino acylated transfer RNA molecules (Hoagland *et al.*, 1957). Four of the sixty-four codons are used as signals to punctuate the code. Aside from specifying the amino acid methionine, the codon AUG is also recognized by a special initiator methionine tRNA to signal the site within the mRNA where translation is to begin (Adams and Capecchi,1966). Three codons, UAA (ochre), UAG (amber) and UGA (opal), do not specify amino acids and are not recognized by any tRNA. Instead they signal the end of translation and are recognized by proteins called releasing factors.

They are the sites where translation stops and the newly synthesized polypeptide is released from the ribosome.

1.3. TRANSFER RIBONUCLEIC ACID

All tRNAs share some common sequence and structural features and can be folded into a compact L-shaped configuration (Holley et al., 1965; Rich and RajBhandary, 1976) (figure 1). Transfer RNAs contain a large number of unusual bases such as dihydrouracil, pseudouracil, 4-thiouracil, methyl or dimethyl guanine and methyl adenine, which are normally not found in other RNA species. These bases are generated by enzymatic modification after transcription and are found in the loop region of the tRNA structure. Their functions are still not fully understood (reviewed in Singer and Kroger, 1979). Nishimira (1979) suggests that the modified nucleosides in the anticodon region may stabilize and enhance certain anticodon-codon base pairing interactions or act as wobble bases. They might also be involved in stabilizing the conformation of the tRNA or its binding to the ribosome, or might enhance its resistance to degradation by ribonuclease or the specificity of recognition of aminoacyl-tRNA synthetase.

All tRNAs contain four stems: the acceptor stem, the D stem, the anticodon stem, and the TψC stem. In some species, an extra arm 3 to 15 bases long may be present between the TψC stem and the anticodon stem. The anticodon loop contains the anticodon which recognizes and base pairs with codons during transcription. The end terminal CCA region of the acceptor stem is the site where the corresponding amino acid is attached by aminoacyl synthetases (reviewed in Saks *et al.*, 1994). The amino acyl tRNAs read the triplet codon on the mRNA by base pairing with the complementary tRNA anticodon loop, and sequentially insert amino acids onto the growing peptide.

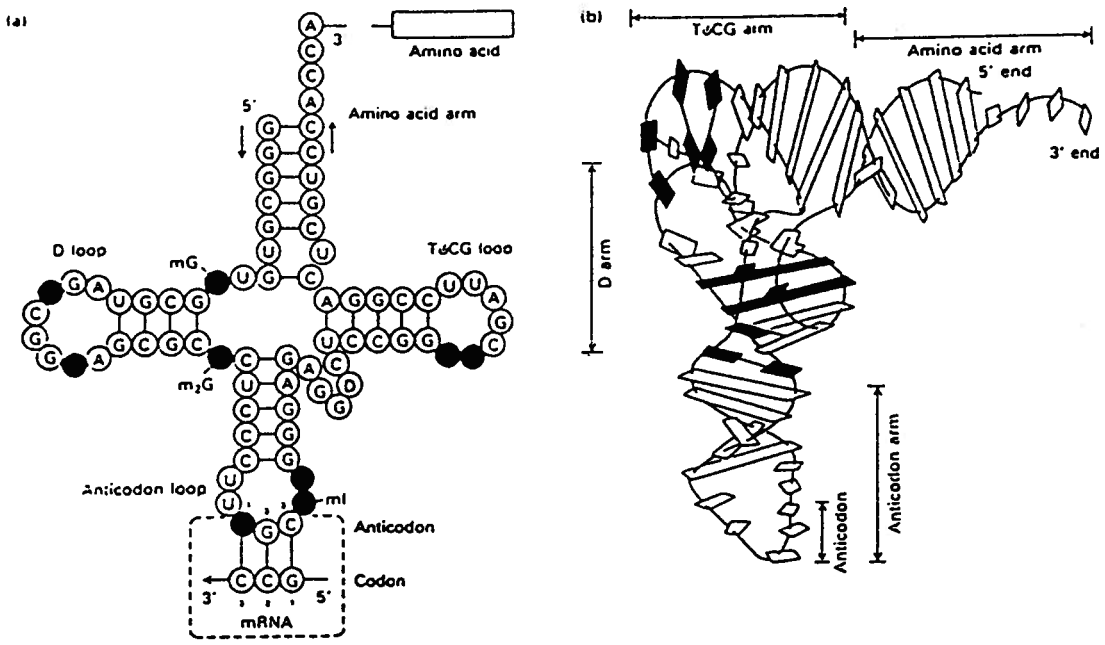


FIG 1: The Structure of Transfer RNA

a. Primary structure of yeast phenylalnine tRNA

b. The compact L-shape molecule of tRNA

Taken from Darnell et at (1986), p.112

1.4 MUTATIONS

A mutation is a change in any DNA base pair and can occur in many forms: substitution, deletion or insertion. Within coding regions, synonymous mutations represent third position substitution between codons specifying a single amino acid; they do not affect the phenotype of the organism, and are often not detected. Non-synonymous mutations are alterations that cause an amino acid replacement in the encoded protein. The resulting polypeptide product is often still functional or partly functional. Insertions and deletions are often deleterious since they often change the reading frame on the messenger RNA. This produces a protein with an aberrant amino acid sequence beyond the site of the frameshift, giving rise to a dysfunctional product. However, when the number of inserted or deleted bases is a multiple of three, the reading frame is not disturbed, and the resulting protein contains either fewer or extra amino acids at the site of the deletion or insertion.

Nonsense mutations are usually recovered less frequently than the other mutations (Garen, 1968). This type of mutation changes an amino acid specific codon to a chain terminating codon, and results in premature termination of translation and production of a truncated polypeptide product. The length of the polypeptide translated depends on the position of the mutation within the gene. Incomplete polypeptide fragments generated by nonsense mutations are usually non-functional because they do not contain all the essential structure required for activity. Since the products of nonsense mutations are usually non-functional, they are very useful in the elucidation of biochemical pathways and protein functions.

1.5 SUPPRESSION

Reversion of a mutation in a gene is often a result of a back mutation to specify the original amino acid. In other cases, it involves second site mutations which can be either intragenic or intergenic. Intergenic suppression can be a result of missense or nonsense suppression.

A missense mutation can be suppressed by a second missense mutation within the same gene (Hill, 1975). When the first missense mutation alters the three dimensional structure of the protein, which is essential for its activity, a second missense mutation may restore the three dimensional structure and hence the biological activity of the protein. Missense suppression is usually inefficient and only partial in its restoration of function. Therefore it is not commonly used in genetic analysis.

In nonsense suppression, a mutant tRNA molecule has an anticodon complementary to a chain terminating codon (reviewed in Lewin, 1974). The mutant tRNA then inserts a functional amino acid at the site of the nonsense mutation to prevent premature termination. The mutation is suppressed and the wild type phenotype will be restored. Suppressors which utilize the UGA, UAA, and UAG stop codons are known as opal, ochre and amber suppressors, respectively (Hirsh, 1971; Garen, 1968; Engelhardt *et al.*, 1965).

The nature of opal suppression is still not fully understood, since its mode of action is different from the model proposed for the other nonsense suppressors (Hirsh, 1971; Hirsh and Gold, 1971). The tryptophan tRNA suppressor is not mutated at the anticodon region but at position 24 of the tRNA, where a G is replaced by an A. This affects the three dimensional structure of the tRNA, which allows moderate suppression of the opal UAG codon. Normal tryptophan tRNA with anticodon CCA can also read the opal stop codon UGA but at a much lower

frequency.

The ochre suppressor is much weaker than the other two suppressors (Garen, 1968). The ochre codon UAA is the most frequently used stop codon *in vivo* and its suppression is clearly deleterious. Cells that carry the ochre suppressor usually grow poorly and therefore an efficient ochre suppressor cannot be isolated.

1.6 AMBER SUPPRESSION

The amber suppressor system is the most widely used and best understood nonsense suppressor system. The suppression of amber mutations is an intergenic nonsense suppression which occurs at the level of translation of mRNA (Engelhardt *et al.*, 1965). The codons UAU and UAC specify the amino acid tyrosine, whereas the amber stop codon UAG specifies termination of messenger RNA translation. In a non-permissive system (one lacking an amber suppressor tRNA), a change in the third base of the tyrosine codon from a U or a C to a G results in premature termination of translation which gives rise to an incomplete and usually non-functional protein product. However, in a permissive system, the minor species of the tyrosine tRNA is mutated in the anticodon from GUA to CUA (the amber suppressor). It can now recognize the UAG stop codon and insert tyrosine at that position, preventing termination. A complete protein product is then formed (Goodman *et al.*, 1968).

In *E. coli*, amber suppressors can be derived from single base mutations in the anticodon region of several different species of tRNA. They can be formed by mutating the third base in the anticodon of glutamine (CUC), glutamic acid (CUG) and lysine (CUU) tRNAs, or the second base of leucine (CAA) and serine (CGA)

tRNAs. However, amber mutants formed by a mutation in the first base position of the tyrosine anticodon (su3) are most widely used and understood. The system utilizes two species of genes which code for tyrosyl-tRNA, a major and a minor one. The major gene is responsible for the synthesis of most of the tyrosyl-tRNA. The minor one consists of two copies of tyrosyl-tRNA genes and either one or both of them is mutated to form a suppressor (Russel et al., 1969). In this way, the system still has an abundant amount of tyrosyl-tRNA for normal translation. The minor species is mapped close to the attachment site of phage 80; therefore it can be packaged into the phage to promote excess synthesis of the minor tRNA (Abelson *et al.*, 1970; Smith *et al.*, 1970). Lewin (1974) summarized the efficiency of 12 amber suppression systems and concluded that the amber suppressor su3 showed the most efficient suppression as measured in the suppression of four different genes (T4 head protein, alkaline phosphatase, β-galactosidase and ornithine transcarbamylase).

Amber suppression has been widely studied and utilized in *E. coli* for analyzing gene expression. Stretton and Brenner (1965) used the amber system to demonstrate that genes and proteins are colinear. Abelson *et al.* (1970) and Smith *et al.* (1970) isolated mutants of tyrosyl-tRNA to study the relationship between sequence change and the functional defects in the molecule. They were able to draw conclusions regarding the structural and functional roles of particular sequences of tRNAs.

Amber suppression provides a convenient genetic system by which the expression of an amber mutated gene can be turned "on" or "off" easily (Gesteland *et al.*, 1967). This provides a way for conditional mutants in an essential gene to be isolated. Suppression competes with chain termination. Each suppressor gene is characterized by the relative frequency of amino acid insertion compared to termination, which ranges from less than 1% to about 60% (Smith *et al.*, 1966).

1.7 ARCHAEBACTERIA

Archaebacteria are a group of organisms that exhibit prokaryotic-like cell structure and organization but at the same time possess a number of eukaryotic features (Dennis, 1986). The discovery and studies of archaebacteria provide us with a new perspective on early events in the evolution of cells, and help us to understand better the universal ancestor and to develop a more accurate concept of eukaryotic origins. Before the recognition of the archaebacterial kingdom, life on earth was divided into two primary kingdoms: the eukaryotes and the prokaryotes, which includes the eubacteria and the archaebacteria. The archaebacteria are now believed to be more closely related to the eukaryotes (Woese, 1981), and comprises one of the three newly defined domains: the eukaryotes (Eucarya), eubacteria (Bacteria) and archaebacteria (Archaea) (Woose *et al.*, 1990).

Archaebacteria propagate at biological extremes of temperature, pH and salt concentration. The group is diverged into two branches: the methanogenic-halophilic and the sulfur-dependent thermophilic branch. The former is composed of groups with two distinct phenotypes: anaerobic methane producers and aerobic halophiles. Halophiles grow at slightly elevated temperatures of 35^0C to 50^0C and salt concentrations of 1.5 to 5M NaCl. They have a sequence complexity comparable to that of*E. coli,* with a high G+C content and their genome sizes range between $5x10^5$ to 10^7 bp (Moore and McCarthy, 1969a). In *Halobacterium halobium,* the G+C content of the chromosomal DNA and the satellite DNA are 66 to 68 mole percent and 57 to 60 mole percent, respectively (Moore and McCarthy, 1969b). Halophiles also possess a restriction-modification system similar to those found in eubacteria (Daniels and Wais, 1984). Their metabolism and physiology are similar to those of eubacteria, with some unusual features such as the synthesis of isoleucine from pyruvate and acetyl-CoA instead of from threonine (Eikmanns and Thauer, 1984).

The structure and catalytic properties of many of their enzymes closely resemble those of either the eubacteria or the eukaryotes.

The halophiles possess many unique genetic characteristics. The chromosomal and extrachromosomal DNA of *H. halobium* are genetically and physically very unstable. Plasmid rearrangements are frequent and complex, and insertions and deletions occur at high frequency. This instability is due to the presence of repetitive sequences and abundant insertion elements. There are at least 500 repetitive sequences in *H. halobium* (Doolittle, 1985), and there are also abundant multiple repetitive sequences in *Haloferax volcanii.*

Comparative analysis of both 5S rRNA and 16S small subunit RNA shows that archaebacteria form a unique and coherent phylogenetic group as reviewed in Dennis (1993). Analysis of the 16S rRNA shows that archaebacteria appear to be more closely related to the common ancestor than either the eubacteria or the eukaryotes (Woese *et al,* 1983). The RNA polymerase found in archaebacteria most closely resembles the eukaryotic RNA polymerase II and III in amino acid sequence. Only a single polymerase has been found in any archaebacterium (Zillig *et al,* 1985) whereas eukaryotes possess three nuclear RNA polymerases.

In archaebacteria, three rRNAs are present: 5S, 16S, and 23S. The ribosomes dissociate into two components: 30S and 50S. Ribosomal RNA sequences are extensively diversified within the archaebacteria; however, there is a clear distinction between those of the archaebacterial kingdom and those of eubacteria and eukaryotes.

Although the general secondary structure of archaebacterial tRNA is similar to the eubacterial and eukaryotic tRNAs (Gupta, 1985), they possess many unique structural details. The archaebacterial tRNA genes lack the 3'-CCA terminal end

(Wich *et al,* 1984; Hui and Dennis, 1985). This sequence is added as a post transcriptional modification by tRNA terminal transferase. The consensus TTAA motif of all archaebacterial promoters appear to be related to the TATA box of the eukaryotic RNA polymerase II promoters (Reiter *et al,* 1990; Thomm and Wich, 1988; Thomm *et al,* 1989).

Archaebacterial transfer RNAs have a characteristic pattern of post-translational modification which is distinctly different from the corresponding eubacterial and eukaryotic patterns. The modified bases T and m^7G found commonly in eubacteria and eukaryotes are absent in all archaebacteria examined (Gupta and Woese, 1980). Modified nucleosides present in the *H. volcanii* tyrosine tRNA include pseudouridine (Ψ), 1-methylpseudouridine(mΨ), 2-methylcytidine (Cm), 5-methylcytidine (m^5C) and 1-methylguanosine (m^1G) (Gupta, 1984). At position 54, instead of the nucleoside T found in most eubacterial and eukaryotic tRNAs, pseudouridine is present in most archaebacterial tRNAs. In the tyrosine tRNA of *H. volcanii,* it is modified to $m^1\Psi$. 1-Methylpseudouridine and T have similar molecular profiles and base pairing properties and could be an example of an evolutionary convergence of structures (Gupta, 1985). The modified nucleosides Ψ and $m^1\Psi$ could be involved in binding tRNAs to ribosomes (Sprinzl *et al.,* 1976). A methylated G (m^1G) is present at position 37. Nishirura (1979) proposed that the modified base right next to the anticodon may help stabilize the codon-anticodon interaction. At position 56 of all examined archaebacterial tRNAs, the modified base methylated cytosine Cm is present. Cm may also be present at position 32. Position 57 of many *H. volcanii* tRNAs consists of a methylated inosine, which is also a unique characteristic of archaebacterial tRNAs.

With the increasing interest in the study of the molecular biology of the archaebacteria, new genetic tools and selection systems are needed for the analysis of

gene structure, function and regulation. The halophilic archaebacterium *H.volcanii* was discovered by Benjamin E. Volcani and was later isolated and studied (Mullakhanbhai and Larsen, 1975). The optimum sodium chloride requirement of the organism is 1.7M, which is close to that found in the Dead Sea. They have a high tolerance for magnesium chloride and are basically disc-shaped; however, the size and shape of the cells vary with the culture conditions and from cell to cell in the same culture. The cells are very fragile and are easily ruptured by mechanical treatments (Muriana,*et al*, 1987) and can be lysed in a hypotonic solution. The DNA has a high GC content (≈63%), which is characteristic of a halobacterium. The cells of *H. volcanii* are orange to red in color due to carotenoids, and have a characteristic odor.

The *H.volcanii* DS2 genome consists of a 2920 kbp chromosome and four plasmids: a 690 kbp pHV4, a 442 kbp pHV3, a 86 kbp pHV1, and a 6.4 kbp pHV2. By ethidium bromide treatment, Charlebois and co-workers (1987) cured the pHV2 plasmid from DS2 to generate a new strain, WFD11. This strain has no visible difference in growth rate or phenotype compared to DS2. It transforms efficiently with the plasmid pHV2 and an artificial construct, pHV2δ93. Using the WFD11 strain, a PEG-mediated spheroplast transformation system was derived (Cline et al., 1989). The efficiency of uptake and expression was comparable to the efficiency of transfection of *H. halobium* with phageH DNA (Cline and Doolittle, 1987). Spheroplasts are generated by addition of EDTA to chelate Mg^{2+} ions. The cells regenerate when Mg^{2+} levels in the growth medium are returned to normal.

Archaebacteria are insensitive to most commonly used antibiotics (Boch and Kandler, 1985). They are found to be sensitive to mevinolin and novobiocin. Spontaneous mutants of *H. volcanii* resistant to the two antibiotics were isolated. This made the development of vectors with selectable markers possible (Holmes

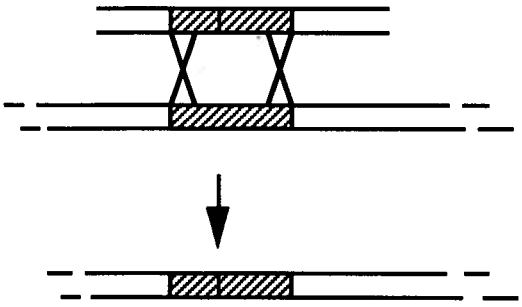
and Dyall-Smith, 1991; Holmes *et al.*, 1991; Lam and Doolittle, 1989). Novobiocin inhibits the activity of eubacterial DNA gyrase by binding to the GyrB subunit and blocking the access of ATP to its binding site on the subunit (Mizuuchi et al, 1978). It is thought that halobacteria are similarly inhibited (Holmes and Dyall-Smith, 1991). Resistant *H. volcanii* have been shown to produce a gyrase that binds novobiocin less avidly (Thiara and Cundliff, 1988). Mevinolin is an inhibitor of the enzyme 3 - hydroxyl - 3 methylglutaryl coenzyme A reductase (Cabrera *et al*, 1985), which is essential in the mevalonate pathway for synthesis of isoprenoid lipids (Kates *et al*, 1968). Cells are inhibited due to decreased mevalonate availability instead of a generalized toxic effect. With the development of an efficient transformation system and the availability of selectable markers, shuttle vector systems were developed (Lam and Doolittle, 1989; Holmes *et al.*, 1991). This provides a convenient system to move DNA back and forth between the *H. volcanii* and *E. coli* systems and greatly simplifies DNA propagation and sequence manipulation.

The shuttle vectors contain the origin of replication in plasmids isolated from different strains of *H.volcanii*, such as pHK2 from strain Aa 2.2 (Holmes *et al*, 1991) and pHV2 from DS2 (Lam and Doolittle, 1989). This provides the essentials for replication and maintenance in the *H. volcanii* system. The selectable antibiotic resistant genes (mev^r or nov^r) are present so that transformants can be isolated efficiently. Fragments of *E. coli* plasmids constitute the rest of the vector, providing the necessary sequences for plasmid selection and maintenance in *E. coli*. A wide selection of shuttle vectors are now available. The newer versions are considerably reduced in size so that larger fragments of DNA can be transformed efficiently (Bissonette and Dennis, unpublished).

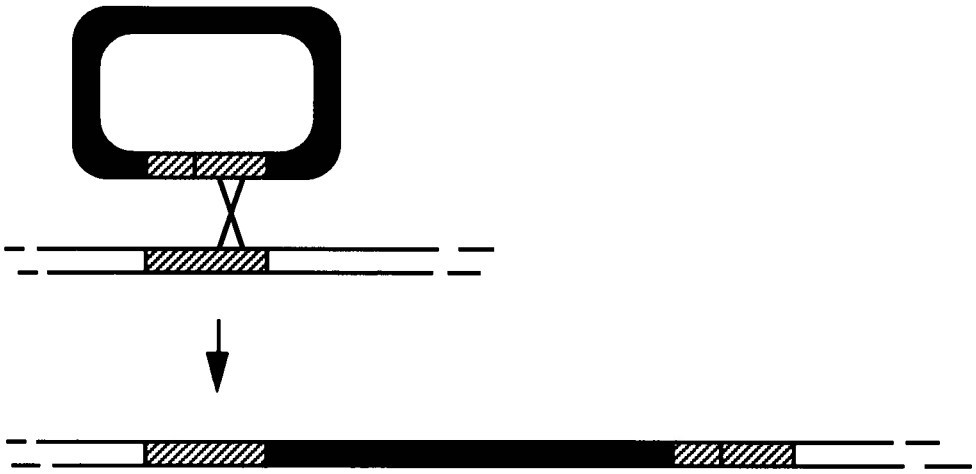
The organism *H. volcanii* was chosen for study because an efficient plasmid transformation and selection system was already made possible with a number of

cloning vehicles. It seems to be genetically more stable than other members of the halophiles due to the presence of fewer insertion sequences (Doolittle, 1985). The genome of *H. volcanii* DS2 has been collected as minimally overlapping fragments of about 36 kbp produced by partial digestion with *MluI* (Charlebois *et al*, 1991). The collection covers 96% of the whole genome and is available as cosmid clones (Cohen *et al.*, 1992). Furthermore, *H. volcanii* grows readily on defined basal salt media with glycerol and succinate as carbon energy source. Since this species is prototrophic, auxotrophic mutants can be readily isolated.

A natural system of genetic exchange (Mevarech and Werczberger, 1985) and gene replacement (Krebs*et al*, 1993) in halobacteria has been discovered. Genetic transfer between *H. volcanii* cells requires cell-to-cell contact and is insensitive to DNase. Transfer is bi-directional and believed to be through cytoplasmic bridges between the two participants of a mating event (Rosenshine and Mevarech, unpublished result cited in Dennis, 1993). Gene replacement is achieved by DNA uptake and integration by homologous recombination of non-replicating DNA into the chromosome (reviewed in Dennis, 1993). Linear DNA can be recombined into the chromosome by two crossing-over events, resulting in gene replacement (Fig 2a), whereas circular DNA is integrated into the chromosome through a single cross-over event, generating a tandem duplication (Fig 2b) (Lam and Doolittle, 1989). Mutant sequences along with a selectable marker on a circular molecule can be put into the chromosome. The clones are then transferred to non-selective media, and the chromosome will readily undergo a second recombination event to expel one of the duplicate sequences. Ideally, in 50% of all cases, the original inserted DNA fragment will be excised. In the other 50% of the cases, the wild type sequence will be excised to produce the replacement. Krebs and co - workers (1993) had successfully utilized this method to introduce deletions into the chromosomal *bop* gene of *H.*



a. LinearDNA recombined into the chromosome by two cross-over events



b. Circular DNA recombined into the chromosome by a single cross-over event

FIG 2. GENE REPLACEMENT IN *Haloferax volcanii*

Products of homologous recombination between a chromosomal sequence and a linear or circular DNA fragment. Homologous DNA sequences are represented by the shaded area. Chromosomal DNA is represented by broken lines. The positions of recombination are indicated by crosses. This figure is adapted from Dennis, 1993

halobium. The *bop* gene activity of the isolated mutants ranged from 0% to 56% activity as compared to the wild type.

1.8 THE EXPERIMENTAL APPROACH

To test whether amber suppression can occur in *H. volcanii* , the tyrosine transfer RNA was cloned and its anticodon region changed from GTA to CTA (RNA sequence GUA to CUA) by site-directed mutagenesis, making it complementary to the amber stop codon UAG. The putative amber suppressor was then tested for its ability to recognize the amber stop codon and to restore wild type function in an amber mutated gene. Originally, the uracil auxotrophy gene was chosen as the candidate for testing the ability of the altered tRNA to suppress amber mutations since it has a convenient positive selection system for auxotrophs (Kondo *et al*, 1991). However, after several attempts to clone the gene and reviewing the results of other experiments performed by Dennis (unpublished), we concluded that the *H. volcanii ura*$^-$ mutants that we obtained from the Doolittle lab exhibit partial activity in the mutant protein and therefore cannot be used in the cloning of the gene.

The *hisC* gene of *H. volcanii* (Conover and Doolittle, 1990) was then used for testing the amber suppression system. It contains an open reading frame which encodes histidinol-phosphate aminotransferase, the eighth enzyme of the histidine biosynthetic pathway. By generating an amber mutation in the gene and then putting the two (*his*(Am) gene and the putative amber suppressor tRNA) together in the *H. volcanii* system, we tested for the possibility of amber suppression in *H. volcanii*.

MATERIALS AND METHODS

2.1 BACTERIAL STRAINS, PLASMID CONSTRUCTION AND OLIGONUCLEOTIDE SEQUENCES

The bacterial strains, plasmid constructions and oligonucleotide sequences that were used are described in Table 2.

2.2 MEDIA AND CULTURE CONDITIONS

All *E. coli* strains were grown either in YT media (5g/l Bacto-yeast extract, 8g/l Bacto-tryptone, 5g/l NaCl, pH7.5), 2xYT or minimal salts (M9) media (6g/l Na_2HPO_4, 3g/l KH_2PO_4, 0.5g/l NaCl, 1g/l NH_4Cl) supplemented with glucose (0.2%), $MgSO_4$ (2mM), $CaCl_2$ (0.1mM) and thiamine (0.5µg/ml). Solid media were prepared by addition of Bacto-agar (15g/l). All strains were grown at 37^0 C. When required, antibiotic concentrations used were: ampicillin (100µg/ml) and kanamycin (50µg/ml).

Haloferax volcanii was grown in the basal salt medium SWG (3.32M NaCl, 0.1M $MgSO_4.7H_2O$, 0.08M $MgCl_2.6H_2O$, 0.07M KCl, 6.8mM $CaCl_2.2H_2O$, 4.9mM NaBr, 2.4 mM $NaHCO_3$) supplemented with 0.5% yeast extract (Oxoid). When minimal media were prepared, the following components were added to 1L of the salt media instead of yeast extract: 5 ml 1M NH_4Cl, 45 ml of 10% glycerol, 5 ml of 10% sodium succinate, 1 ml of trace elements and 2 ml of 0.5M K_2HPO_4(Mevarech and Werczberger, 1985). Solid media were prepared by addition of 15g/l agar technical (agar no. 3; Oxoid). Histidine or arginine supplements were added at a concentration of 50µg/ml when needed. All strains were grown at either 37^0C or 42^0C. The antibiotics mevinolin and novobiocin were added at concentrations of 50µM and 0.2 µg/ml respectively when needed.

TABLE 2: BACTERIAL STRAINS, PLASMID CONSTRUCTIONS AND OLIGONUCLEOTIDE SEQUENCES

	Strain	Description
E. coli	DH5α	*F- recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 λ-*
	HB2151	*K12 ora δ(lac-pro) thi/F' proA⁺B⁺ lacZ δM15*
	JM101	*supE thi δ(lac-proAB), [F' traD36 proAB lacI^qZδM15]*
	RZ1032	Zbd-279*[lysA(61-62)], dut1, ung1, thi1, rel1, supE44,Tn*10
H. volcanii	DS2	wild type *Haloferax volcanii* consisting of a 2920 kbp chromosome and 4 plasmids: pHV1-4
	WFD11	strain DS2 lacking the plasmid pHV2
	WR256	mutagenised WFD11 *his⁻ arg⁻*

Plasmid	Description
pT4C	2.7 kbp *Sac*I-*Mlu*I fragment containing *his*C⁺ gene in *Sma*I site of pGS18 obtained from the Doolittle lab.
pHC	2.7 kbp *Bam*HI-*Eco*RI fragment containing *his*C⁺ gene subclone from pT4C in pGEM7(-)
pHC12	a derivative of pHC containing the mutation TAC to TAG in codon 12 of *his*C
pT1	4.5 kbp *Sma*I fragment containing the *H. volcanii* tyrosine tRNA gene in pGEM7(-)
pT2.1	800bp *Sma*I-*Eco*RI fragment containing the *H. volcanii* tyrosine tRNA gene in pGEM7(-)

pT2.2	800bp *Sma*I-*Eco*RI fragment containing the *H. volcanii* tyrosine tRNA gene with an altered anticodon GUA to CUA
pT3	1.5 kbp SmaI fragment in pGEM7(-) containing the putative amber suppressing tyrosine tRNA gene
Oligonucleotide	Description
O1	TCCGCTCTCCCCGATTT/C a minus strand sequence complementary to position 73 to 57 within the tyrosine tRNA gene
O2	AGAGCAGCCGACTGTAG a plus strand sequence identical to position 21 to 37 within the tyrosine tRNA gene
O3	CTGCTCAAACCGGCTCG a minus strand sequence complementary to the region 17 bp 3′ downstream of tyr tRNA
O4	CGATCTAGAGTCGGCTGCTCT a minus strand sequence complementary to position 41 to 21 within the tyrosine tRNA gene except at position 34, where a G is present instead of a C
O5(JMH)	ACGCTCCCTAGGTACCCGGCCG a plus strand sequence identical to position 26 to 47 within the *his*C gene except at position 36, where a G is present instead of a C
O6(JSH)	TCGGTCGTCGCGTCCCCAAC a plus strand sequence identical to position 70 to 89 within the *his*C gene

O7(JSH2)	TGGGCGGTCTTCGGGTAGAC
	a minus strand sequence complementary to
	position 200 to 180 within the *hisC* gene

2.3 GENERAL TECHNIQUES OF MOLECULAR BIOLOGY

General recombinant DNA techniques were carried out according to Sambrook *et al.* (1982) unless otherwise specified.

2.3.1 PREPARATION OF PLASMID DNA

Small scale preparation of plasmid DNA was done by the alkaline lysis method (Sambrook *et al.*, 1982) or one-step miniprep method (Chowdhury, 1991). Large scale DNA was prepared using alkaline lysis (Sambrook *et al.*, 1982), with the following modifications: the supernatant was not filtered through layers of cheesecloth. The final DNA pellet was dissolved in 97% CsCl (97g/100ml dH_2O) and banded in a CsCl-ethidium bromide gradient at 50,000 rpm overnight. The DNA obtained after removal of EtBr was diluted in three volumes dH_2O and then precipitated with 95% EtOH.

2.3.2 PREPARATION OF DOUBLE STRANDED DNA FOR SEQUENCING

Double stranded DNA was either prepared as described in Saunders and Burke (1990) using CsCl or with the Magic miniprep DNA purification system supplied by Promega. Reactions were carried out according to manufacturer's protocol.

2.3.3 RESTRICTION ENDONUCLEASE DIGESTION OF DNA

Restriction enzymes used were purchased from Pharmacia Inc., Bethesda

Research Laboratories (BRL) or New England Biolabs. Digestions were carried out according to the instructions of suppliers.

2.3.4 GEL ELECTROPHORESIS

Samples of DNA were separated by electrophoresis on agarose gels (0.7% or 1%) in TBE buffer (89mM Tris, 89mM boric acid, 2.5mM Na_2EDTA) in the presence of 0.25 mg/ml ethidium bromide.

2.3.5 DNA RESTRICTION FRAGMENT PREPARATION

Bands of restricted DNA, stained with ethidium bromide, were excised from agarose gels and recovered using Sephaglas BandPrep Kit (Pharmacia)

2.3.6 OLIGONUCLEOTIDE PURIFICATION

Oligonucleotides were purified as described in Sawadogo and Dyke (1991) and the concentrations were determined by spectrophotometry.

2.3.7 DEPHOSPHORYLATION OF IDENTICAL COHESIVE TERMINI OF VECTOR DNA

Identical cohesive termini of vector DNA were dephosphorylated with shrimp alkaline phosphatase (SAP) (United States Biochemical) by using 3u of enzyme per microgram of DNA. The reaction was incubated in SAP buffer (20mM Tris-Cl pH8.0, 10mM $MgCl_2$) for 1 hour at 37^0C to prevent religation. The enzyme was then heat inactivated at 65^0C for 15 minutes.

2.3.8 LIGATIONS

Cohesive-end ligations were incubated at 14-16^0C overnight or 2 hours at room temperature.

For blunt-end ligations, incubations were at room temperature overnight or 2 hours with 5% PEG8000.

2.3.9 COMPETENT CELLS AND TRANSFORMATION

Competent cells (DH5α or HB2151) were made by treatment with $CaCl_2$ (Sambrook *et al, 1982*). Frozen competent cells were prepared as above and resuspended in ice-cold 100mM $CaCl_2$ containing 15% glycerol. The suspension was incubated on ice for 1 hour and then aliquoted into 100µl stocks. These were then frozen in dry ice and stored at -70^{0}C until needed. Transformation of plasmid DNA was carried out by incubating competent cells with DNA on ice for 30 minutes. The cells were then heat shocked at 37^{0}C for 2 minutes, plated on selective media and incubated for 12-16 hours.

2.3.10 PREPARATION OF SINGLE -STRANDED DNA

Single stranded DNA template can be made from a host carrying a phagemid by superinfection using a helper phage. The plasmid pGEM7(-) can be used as a phagemid since it contains the f1 origin of replication. A single colony of freshly grown HB2151 on M9 plates containing the plasmid pGEM7(-) with the appropriate inserts was inoculated into 2xYT media. The culture was grown to mid-exponential phase. It was then infected with either phage R408 or M13K07 and incubated for 6 hours with good aeration. Cells were then spun down and the phage particles and DNA were precipitated in 4% PEG8000 and 0.7M NH_4OAc. The pellet was resuspended in TESDS(100mM Tris, 10mM EDTA, 0.1%SDS) and incubated with proteinase K for 30 minutes. The sample was then extracted with phenol and chloroform until no protein interface was observed and then precipitated and washed with ethanol.

2.3.11 GENERATION OF DELETIONS BY EXONUCLEASE

Exonuclease deletion was performed as described in the Promega protocols and application guide(1991), p90-98, at 32.5^{0}C. Samples were taken every 30 seconds. After treatment with Klenow and dNTPs, the samples were loaded on a 1% agarose gel to determine the efficiency and extent of deletion. The bands were then recovered as described in 2.3.5, the ends were religated to form a circular plasmid and then transformed into DH5α.

2.3.12 SEQUENCING

Sequencing was performed according to the instructions supplied with the Deaza G/A ^{T7} Sequencing Kit (Pharmacia) with the exception that the primer was added to the double stranded DNA template before denaturing in 0.2M NaOH. Furthermore, the template/primer/NaOH mixture was denatured by boiling in a water bath for 5 minutes and then quickly quenched on ice.

2.3.13 LABELING OF OLIGONUCLEOTIDE AND FRAGMENT PROBES

Oligonucleotide probes were end-labeled using bacteriophage T4 polynucleotide kinase (PNK) and [γ-^{32}P]ATP (sp. act. 3000 Ci/mmol, 10μCi/μl). Bacteriophage T4 PNK was inactivated by heating to 65^{0}C for 10 minutes.

Restriction fragments purified as in section 2.3.5 were radiolabeled using the Random Primers DNA Labeling System (BRL). The labeling reaction was carried out at 25^{0}C for 1 hour. The enzyme was then inactivated by addition of 5μl of stop buffer and boiled for 5 minutes in a water bath. Approximately 100ng of DNA fragment was used in each reaction and half of the labeled reaction mixture was used in each Southern Hybridization.

2.3.14 SOUTHERN HYBRIDIZATION

Southern hybridization was carried out either on Hybond paper or using the dried-down agarose gel.

DNA was transferred and probed on Hybond paper as described in the protocol: Blotting and hybridization protocols for Hybond^{TM} membranes supplied by Amersham. The transfer was carried out overnight. Prehybridization was carried out at 40^0C or 65^0C and hybridization at 50^0C for labeled oligonucleotide probes or 65^0C for fragment probe respectively. When oligonucleotide probes were used, the third wash was not carried out.

Hybridization with genomic DNA was done in a dried gel. The 1% agarose gel was dried under vacuum at room temperature for 1 hour and then at 60^0C for another 30 minutes or until the gel is dried. The gel was then denatured for 30 minutes in 0.5M NaOH/0.15M NaCl and neutralized twice for 15 minutes in 0.15M Tris/0.15M NaCl. The gel was hybridized to the probe by heating to the estimated Tm(melting temperature) of the probe for 30 minutes and then cooled slowly down to room temperature. The hybridized gel was then washed with 6xSSC (175.3g/L NaCl. 88.2g/L Na.citrate.$2H_2O$, pH7) to remove any non-specific binding of probe. Autoradiography was then carried out on the membrane or gel.

2.4 MOLECULAR BIOLOGY TECHNIQUES FOR *HALOFERAX VOLCANII*

2.4.1 PREPARATION OF COMPETENT CELLS AND TRANSFORMATION

Competent cells of WFD11 or other strains of halobacteria were prepared by pelleting 1 ml of culture in mid or late exponential phase(O.D.= 0.5-1.5) and then resuspending in 200µl spheroplast generation solution (0.8 M NaCl, 27mM KCl, 50mM Tris-HCl, 15% glycerol, 15% sucrose). The cells can either be used immediately or frozen at -70^0C.

Spheroplasts were formed by addition of 20µl 0.5M EDTA and incubated with

the DNA solution. Then 240µl of HFPEG(6ml PEG600 and 4ml spheroplast generation solution) was added and mixed gently until a homogenous solution was obtained. Cells were regenerated by adding 1 ml of solution R (3.4M NaCl, 175mM $MgSO_4.7H_2O$, 34mM KCl, 7mM $CaCl_2.2H_2O$, 50mM Tris-HCl, 15% sucrose) and grown in SWGR (SWG supplemented with yeast extract and 15% sucrose) for 6 hours before plating on appropriate media(Cline et al, 1989). The plates were incubated at 42^0C for 5-14 days.

2.4.2 PREPARATION OF GENOMIC DNA

The pellet from 3mls of a *H. volcanii* culture was resuspended in 0.4 ml lysis buffer(40mM Tris, 20mM EDTA and 10mg/ml lysozyme), vortexed vigorously and incubated at 37^0C for 30 minutes. Then 0.1 ml of 5% SDS with 100µg/ml RNase was then added and incubated for 5 minutes at 45-60^0C. The clear, viscous solution was then extracted with phenol twice and then with chloroform: isoamyl alcohol once. The aqueous phase was transferred to a fresh tube containing 15µl of 5M NaCl and mixed well. To precipitate the DNA, cold 95% ethanol was added and mixed gently until DNA threads were visible. The DNA was spooled onto a sealed pasteur pipette and rinsed by dribbling 1 ml of 70% ethanol over the pipet tip. The DNA was then dried by standing the tip up for 10 minutes and resuspended by swirling the tip in 0.2ml TE warmed to 37^0C.

2.4.3 PREPARATION OF PLASMID DNA

One milliliter of *H. volcanii* culture was pelleted and resuspended in 100µl of HFNTE (1M NaCl, 50mM Tris, 10mM EDTA, pH8). Cells were lysed by adding 5 µl of 5% deoxycholic acid, followed by 200µl of alkaline lysis solution II (0.2N NaOH and 1% SDS) and solutionIII (294.5 g/L potassium acetate and 115 ml/L acetic acid). It was then centrifuged and the supernatant was recovered. DNA was ethanol precipitated

and resuspended in TE. Proteins were differentially precipitated by adding ammonium acetate to a final concentration of 2M.

2.4.4 SHUTTLE VECTORS FOR *H. VOLCANII*

Shuttle vectors were used throughout the experiments to move DNA back and forth between *E. coli* and *H. volcanii* for genetic manipulation and *in vivo* gene exprssions respectively. The pGOT series of shuttle vectors used are as described in Bissonnette and Dennis (unpublished).

2.5 MUTAGENESIS

Uracil containing single stranded DNA template was synthesized in RZ1032, and prepared as described in 2.3.10. Mutagenesis (Kunkel, 1985; Kunkel *et al*, 1987; Ner *et al*,1988) was carried out with oligo 4 on tyr tRNA gene and with oligo 5 on *his C* gene.

One microgram of uracil containing single stranded DNA template was annealed with 5 pmol of the corresponding phosphorylated oligonucleotide in 10X annealing buffer (100mM Tris-HCl pH8, 500mM NaCl, 100mM $MgCl_2$ and 10mM DTT). The mixture was heated to 75^0C for 10 minutes and cooled slowly to room temperature. Extension and ligation were then performed by addition of one-fifth volume of 5x polymerase mix (100mM Tris-HCl pH8.8, 10mM DTT, 50mM $MgCl_2$, 5mM ATP and 2.5mM each of dATP, dTTP, dGTP, dCTP) and 2U of T4 DNA ligase and 2 u Klenow. The mixture was left on ice for 5 minutes, then incubated at room temperature for 2 hours. Further aliquots of Klenow (2u) and ligase (2u) were added to the mixture and incubated at 37^0C for another 2 hours. One-tenth of the reaction mixture was transformed into *E.coli* DH5α and colonies formed were individually picked and sequenced. Only the G and C sequencing reactions were performed in the

initial screening of the mutagenized clones to avoid redundant sequencing.

2.6. AUTORADIOGRAPH SCANNING

All autoradiographs obtained from Southern hybridizations and sequencing reactions were scanned using a flatbed scanner (Hewlett Packard ScanJet Plus). The images were printed with a scanning program onto a laser printer.

RESULTS

3.1 Cloning of tyrosine tRNA gene

The sequence of the tyrosine tRNA was determined by Gupta (1984), (fig3). To clone the tyrosine tRNA gene, Southern hybridization was carried out on genomic digests of DS2 DNA to detect the gene. Fragments from the region where a positive signal was obtained were then cloned into a vector to obtain a size-fractionated partial library. The library was then rescreened to obtain an individual clone which carries the gene.

Oligonucleotides 1 and 2 were used for detecting for the presence of the tyrosine tRNA gene of *H. volcanii*. Oligonucleotide 1 is complementary to the tRNA sequence (fig 3) at the 3′ end from position 73 to 57, with a degenerate 3′ end at position 57. Oligonucleotide 2 is identical to the RNA sequence from position 21 to 37, which includes the anticodon region. Genomic DNAs from *H. volcanii* DS2 and WFD11 were digested with different restriction enzymes and separated on a 1% agarose gel. Southern hybridization was carried out using radiolabeled oligonucleotide 1 to detect the location of the tyrosine tRNA gene (fig 4). Positive signals were further confirmed by hybridization with oligonucleotide 2. The results obtained from the DS2 and WFD11 DNA digests were identical.

A *Sma*I genomic fragment of about 1.5kbp in length hybridized to both oligonucleotides. To clone the fragment, DNA from this region of the gel (1.4 to 1.6kbp) was eluted, purified, shotgun cloned into the dephosphorylated *Sma*I site of pGEM7(-) and transformed into *E. coli* DH5α. Plasmid DNA from the transformants was analyzed in pools of 4 and screened for the presence of the gene by Southern hybridization using oligonucleotide 2 as probe. Individual transformants from

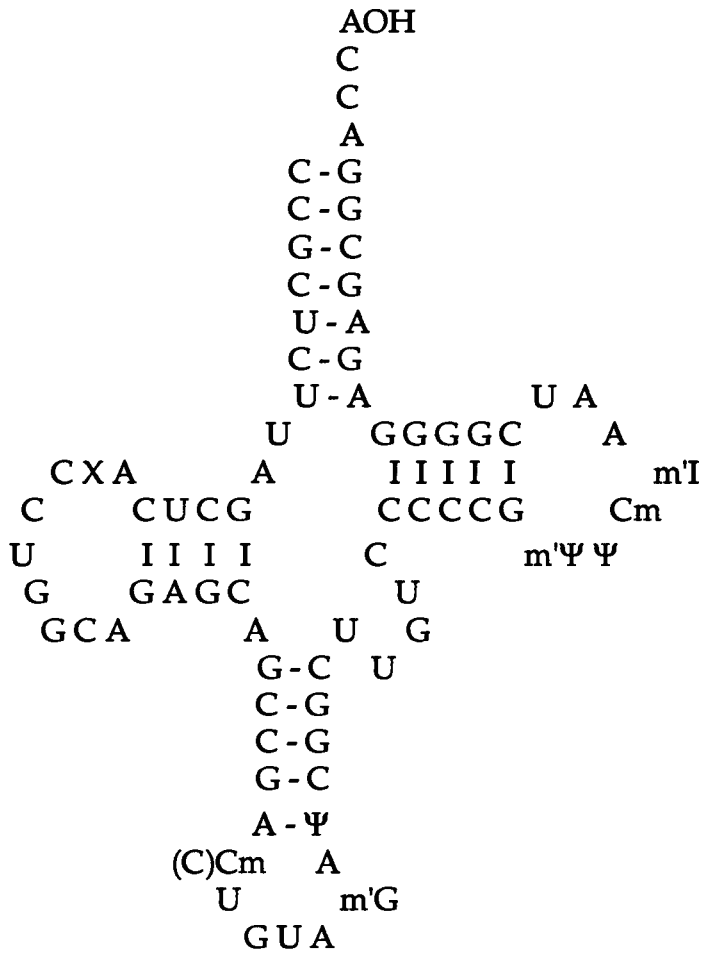


FIG 3. Tyrosine transfer RNA sequence (adapted from Gupta, 1984)

Transfer RNAs of *H. volcanii* were separated by two-dimensional gel electrophoresis and sequenced. Gupta (1984) presented the RNA sequence of 41 *H. volcanii* tRNAs including the tyrosine tRNA as shown. They resemble the general structure and sequence of eubacterial and eukaryotic tRNAs.

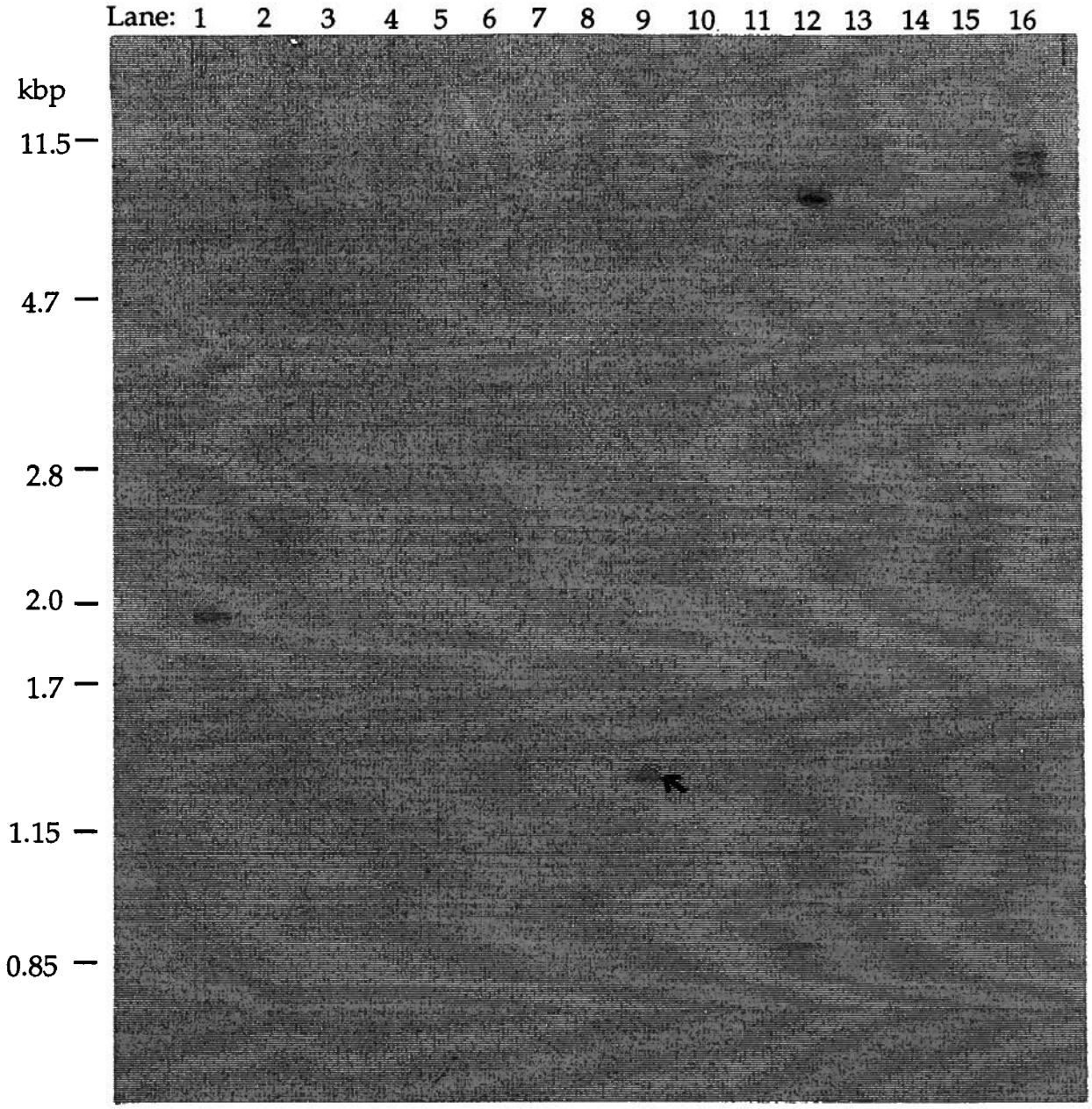


FIG 4: Southern hybridization of restricted wild type genomic DNA to detect the presence of the tyrosine transfer RNA gene

Genomic DNA of wild type *Hvo* DS2 was digested with various restriction enzymes: Lanes: 1, *Mbo*I; 2, *Rsa*I; 3,*Bgl*I; 4,*Hind*III; 5,*Eco*RI; 6,*Bam*HI; 7,*Pst*I; 8 *Sal*I; 9, *Sma*I; 10, *Xho*I; 11, *Taq*I; 12, *Nco*I; 13, *Nar*I; 14, *Msp*I; 15, *Mlu*I;, 16, *Asp*718. Arrow shows the positive signal at 1.5kbp of the *Sma*I digest. Oligonucleotide1 was used as probe.

positive pools were rescreened in order to obtain the correct clone.

3.2 The tyrosine tRNA sequence

The positive clone pT1 was chosen for further study. By restriction mapping (fig 5), the structure of plasmid pT1 was analyzed. When the plasmid was digested with *Sma*I, a 4.5 kbp band and two bands around 1.5kbp were observed. When digested with *Eco*RI, 2.1 kbp and 5.1 kbp fragments were observed. When the plasmid was digested with both enzymes, a 3 kbp vector band, two 1.5 kbp bands, a 0.8 kbp band and a 0.6 kbp band were observed. Southern hybridization showed that the tyrosine tRNA gene is contained in the 1.5kbp *Sma*I, the 5.1kbp *Eco*RI and the 0.8kbp *Eco*RI-*Sma*I fragments. The plasmid pT1 contained three 1.5 kbp fragments in pGEM7(-). The first *Sma*I site was lost since *Sma*I gives blunt ends. The tyrosine tRNA coding region is contained in the middle fragment 38 bp downstream from the *Eco*RI site (fig 6).

The DNA sequence of the tyrosine tRNA gene was determined using oligonucleotide 2 and oligonucleotide 3 as primers (fig 7). At position 15, where the nucleotide is undefined from the published RNA sequence, the DNA sequence contained a G residue. Furthermore, the methylated inosine at position 57 was determined to have come from the nucleotide A. The putative promoter was present as the TTAA box at position -32. The putative terminator was also present as a stretch of Ts followed by As. Upstream consensus promoter elements were also identified at positions -44, -38 and -13. The *Eco*RI site was present as part of the promoter element at position -38. The terminal sequence CAA is absent in the DNA sequence of the gene.

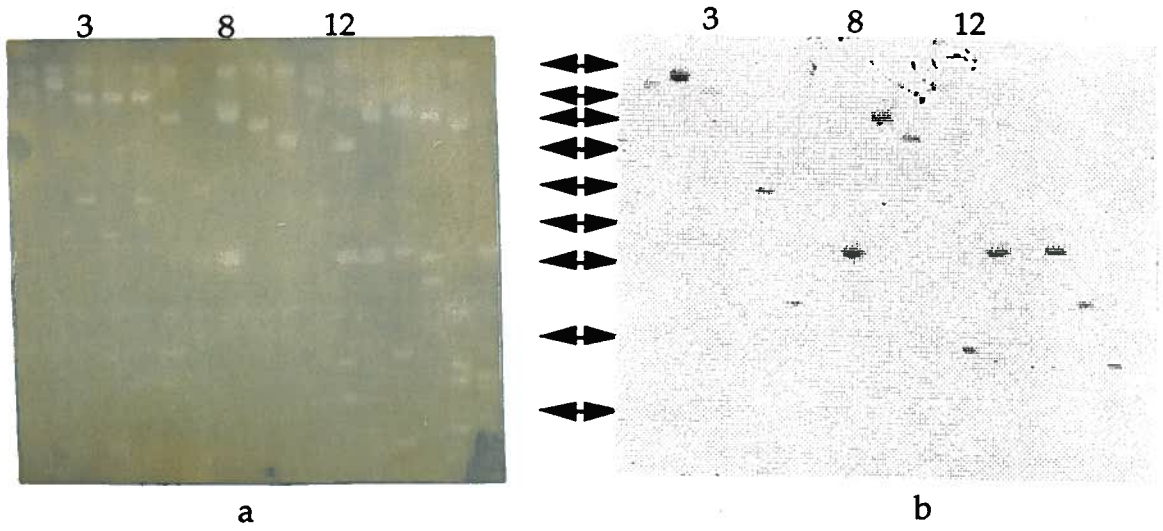


FIG 5: Restriction mapping of plasmid pT1.

(a) Plasmid pT1 was digested with different restriction enzymes and fractionated by electrophoresis. Lanes: 1, uncut; 2, *Asp*718; 3, *Eco*RI; 4, *Mlu*I; 5, *Nco*I; 6, *Sal*I; 7 *Sau*3A; 8, *Sma*I; 9, *Sph*I; 10, *Sst*I; 11, *Pst*I; 12, *Eco*RI+*Sma*I; 13, *Eco*RV+*Sma*I; 14, *Mlu*I+*Sma*I; 15, *Nco*I+*Sma*I; 16, *Sal*I+*Sma*I; 17, *Sau*3A+*Sma*I. It was then transferred to nitrocellulose and probed with ^{32}P labeled oligonucleotide2. The location of the tyrosine tRNA gene within each digest is shown in (b). Size markers (λ DNA cut with PstI) are indicated by arrows from top to bottom: 11.5, 5.1, 4.5, 2.8, 2.0, 1.7, 1.2, 0.8, 0.4 kbp.

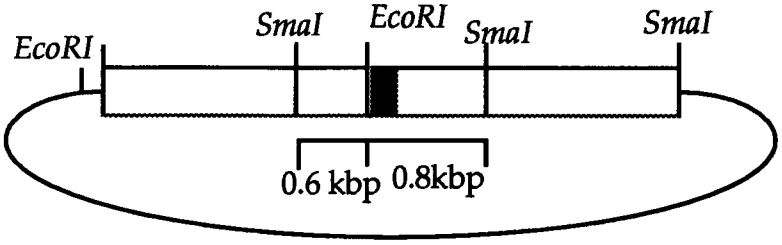


FIG 6 :The plasmid pT1

■ Tyr tRNA gene

The plasmid pT1 contains three 1.5 kbp fragments cloned from genomic *Sma*I digested DS2 DNA. The tyrosine transfer RNA gene is located in the middle fragment 32 bases downstream of the *Eco*RI site, which starts approximately 0.6 kbp downstream of the second*Sma*I site. The first *Sma*I site was lost during religation of blunt ends.

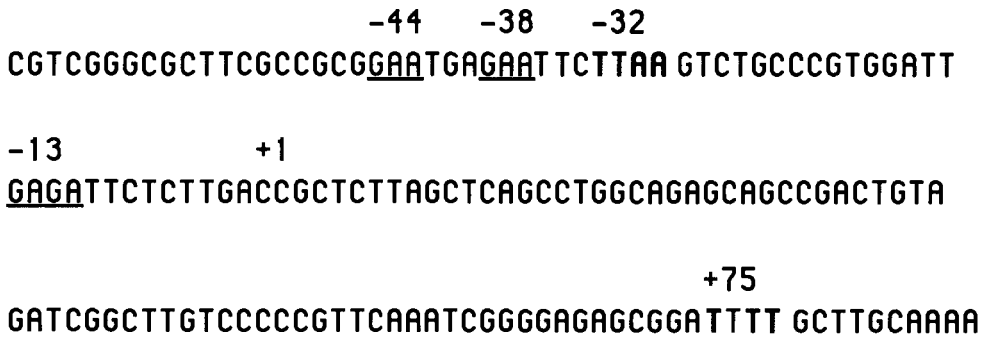


FIG 7: DNA Sequence of the tyrosine tRNA gene

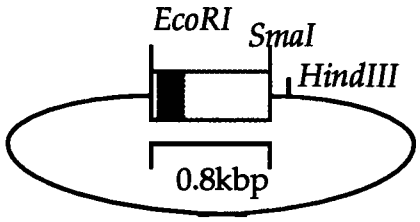
The sequence of the tyrosine transfer RNA gene was determined by sequencing using oligo 2 and 3 as primers. The gene is only 73 base pairs in length. The upstream consensus promoter elements are identified at positions -44, -38 and -13. The promoter sequence TTAA is present at position -32 and the terminator sequence TTTT at position 75.

3.3 Mutagenesis of tyrosine tRNA gene

The 0.8 kbp *Sma*I-*Eco*RI fragment from plasmid pT1 was subcloned into pGEM7(-) (pT2.1)(Fig 8a). The smaller fragment permits sequencing through the whole gene with the universal primer. The possibility of incorporation of wrong nucleotides into the upstream region during site-directed mutagenesis is also eliminated. Site-directed mutagenesis (Smith, 1986) was carried out on pT2.1 using oligonucleotide 4. The anticodon region was mutated from GUA to CUA(fig 9), which in principle should allow the tRNA to recognize the amber stop codon TAG (pT2.2; fig 8a). The mutated 0.8 kbp fragment was then excised using *Hind*III and *Eco*RI and religated with the upstream 0.6 kbp *Sma*I *Eco*RI fragment into the *Hind*III *Sma*I site of pGEM7(-) to reform the entire gene with complete upstream promoter elements. This clone containing the putative tyrosine tRNA amber suppressing gene was designated pT3 (fig 8b).

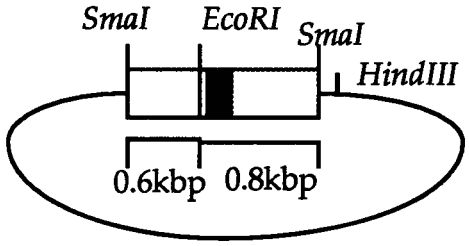
3.4 The *hisC* gene

A clone of the *hisC* gene of *H. volcanii* (Conover and Doolittle, 1990) (fig 10) was obtained as pT4C, which contains a 2.7kbp fragment in pBGS18, a kanamycin resistant derivative of pUC18 (Spratt et al, 1986). Since the vector pGEM7(-) was used throughout the project, the *hisC* gene was recloned into the vector for consistency and convenient manipulation. The plasmid pT4C was cut using *Bam*HI and *Eco*RI and the 2.7 kbp fragment containing the *hisC* gene was ligated into the *Bam*HI-*Eco*RI site of pGEM7(-) to form pHC(fig 11).



a. plasmid pT2.1/pT2.2 containing 0.8 kpb insert in pGEM7(-)

■ Tyr tRNA gene



b. plasmid pT3 containing 1.5 kpb insert in pGEM7(-)

■ Tyr tRNA gene

FIG 8: Plasmid construction of pT2.1, pT2.2 and pT3

Plasmid pT2.1 was constructed by cloning the 0.8 kbp *Eco*RI-*Sma*I fragment from pT1 into the *Eco*RI-*Sma*I site of pGEM7(-). Site-directed mutagenesis was carried out on pT2.1 to change the anticodon region from GTA to CTA, the resultant plasmid was named pT2.2. The mutated 0.8 kbp fragment from pT2.2 was recovered by cutting with *Eco*RI and *Hind*III and religated to the 0.6 kbp *Sma*I-*Eco*RI fragment from pT1. The fragment was cloned into the *Sma*I-*Hind*III site of pGEM7(-) to form pT3.

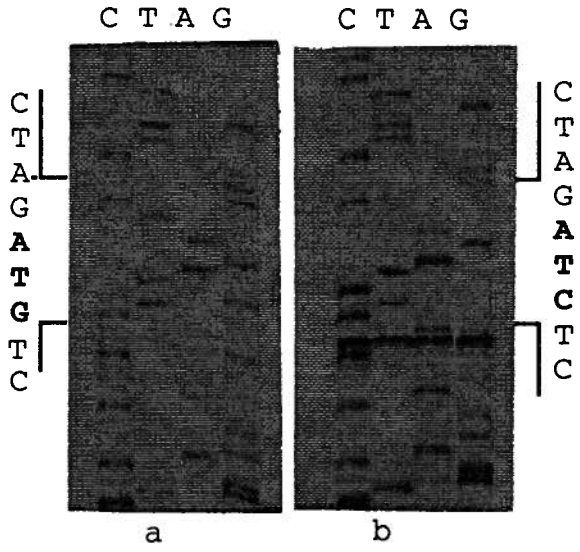


FIG 9: Site-Directed Mutagenesis of tyr tRNA

The two panels are: (a) DNA sequence of the anticodon region of the tyrosine tRNA before site-directed mutagenesis. The DNA sequence of the anticodon is GTA, which give rise to tRNAs that recognize the tyrosine codon UAC or UAU; and (b) DNA sequence of the anticodon region of the tyrosine tRNA after site-directed mutagenesis. The DNA sequence of the anticodon is changed to CTA, which in principle should give rise to tRNAs that recognize the amber stop codon UAG

```
AGTCGTTCGGGCGGCGCTCGGCTGACGGCCGTCGGTCGTCGCG

TCCCCAACCCGACCCCCTACCGCCACGTCCGACCCGGAGTACGCACCCTTAAGAACCGCGACCCGCATTTTCCGACC
+1
ATG CAA CCA CGG GAC CTC TCC GCG CAC GCT CCC TAC GTA CCC GGC CGC GGG ACA GAG
 M   Q   P   R   D   L   S   A   H   A   P   Y   V   P   G   R   G   T   E
GAG GTC GCC CGC GAA CTC GGA ATG GAC CCC GAG GAC CTG ACG AAA CTC TCC TCG AAC
 E   V   A   R   E   L   G   M   D   P   E   D   L   T   K   L   S   S   N
GAG AAC CCC CAC GGC CCG AGT CCG AAG GCG GTC GCC GCC ATC GAA GAC GCC GCG CCG
 E   N   P   H   G   P   S   P   K   A   V   A   A   I   E   D   A   A   P
ACC GTG AGC GTC TAC CCG AAG ACC GCC CAC ACG GAC CTG ACC GAA CGC CTC GCC GAC
 T   V   S   V   Y   P   K   T   A   H   T   D   L   T   E   R   L   A   D
AAG TGG GGC CTC GCA CCC GAA CAG GTG TGG GTG TCT CCC GGC GCG GAC GGC TCT ATC
 K   W   G   L   A   P   E   Q   V   W   V   S   P   G   A   D   G   S   I
GAC TAC CTG ACC CGC GCG GTG CTC GAA CCG GAC GAC CGG ATT CTC GAA CCC GCG CCC
 D   Y   L   T   R   A   V   L   E   P   D   D   R   I   L   E   P   A   P
GGC TTT TCG TAC TAC TCG ATG AGC GCC CGC TAC CAC CAC GGC GAC GCC GTC CAG TAC
 G   F   S   Y   Y   S   M   S   A   R   Y   H   H   G   D   A   V   Q   Y
GAG GTG TCG AAG GAC GAC GAC TTC GAA CAG ACC GCC GAC CTC GTC CTC GAC GCC TAC
 E   V   S   K   D   D   D   F   E   Q   T   A   D   L   V   L   D   A   Y
GAC GGC GAG CGC ATG GTC TAC CTC ACA ACG CCG CAC AAC CCC ACC GGT TCC GTG CTC
 D   G   E   R   M   V   Y   L   T   T   P   H   N   P   T   G   S   V   L
CCG CGG GAG GAA CTC GTC GAA CTG GCC GAG TCG GTC GAA GAG CAC ACG CTC CTC GTC
 P   R   E   E   L   V   E   L   A   E   S   V   E   E   H   T   L   L   V
GTC GAC GAG GCC TAC GGC GAG TTC GCC GAG GAG CCG TCG GCC ATC GAC CTC TTG TCG
 V   D   E   A   Y   G   E   F   A   E   E   P   S   A   I   D   L   L   S
GAG TAC GAC AAC GTC GCG GCC CTG CGG ACG TTC TCG AAG GCG TAC GGG CTG GCC GGC
 E   Y   D   N   V   A   A   L   R   T   F   S   K   A   Y   G   L   A   G
CTC CGC ATC GGC TAC GCC TGC GTG CCC GAG GCG TGG GCC GAC GCC TAC GCC CGC GTG
 L   R   I   G   Y   A   C   V   P   E   A   W   A   D   A   Y   A   R   V
AAC ACG CCG TTC GCC GCC AGC GAG GTC GCC TGC CGC GCC GCG CTC GCC GCG CTC GAC
 N   T   P   F   A   A   S   E   V   A   C   R   A   A   L   A   A   L   D
GAC GAG GAA CAC GTC GAG AAA TCC GTC GAG TCG GCC CGG TGG TCC CGC GAC TAT CTC
 D   E   E   H   V   E   K   S   V   E   S   A   R   W   S   R   D   Y   L
CGC GAA CAC CTC GAC GCG CCG ACG TGG GAA AGC GAG GGC AAC TTC GTC CTC GTC GAG
 R   E   H   L   D   A   P   T   W   E   S   E   G   N   F   V   L   V   E
GTC GGC GAC GCC ACG GCC GTC ACC GAG GCC GCC CAG CGC GAG GGC GTC ATC GTC CGC
 V   G   D   A   T   A   V   T   E   A   A   Q   R   E   G   V   I   V   R
GAC TGC GGG AGC TTC GGC CTG CCG GAG TGC ATC CGC GTC TCC TGC GGC ACG GAA ACC
 D   C   G   S   F   G   L   P   E   C   I   R   V   S   C   G   T   E   T
CAG ACC AAG CGC GCC GTG GAC GTG CTC AAC CGC ATC GTC TCG GAG GTG CCG ACG GCG
 Q   T   K   R   A   V   D   V   L   N   R   I   V   S   E   V   P   T   A
TGA GAGACGACGACACCGGCACGCCCGGCACCGGAAAGACCACGGCGACCGAGCCGGTCGCCGCCGACCTCGACC
end
TCGACGTGGTCCACCTCAACCGACTCGTGAAAGACGAGG
```

FIG 10: DNA and derived amino acid sequences of the *H. volcanii hisC* gene The putative promoter TTAA is identified in bold and the first tyrosine codon (TAC) is underlined. The figure is adapted from Conover and Doolittle, 1990.

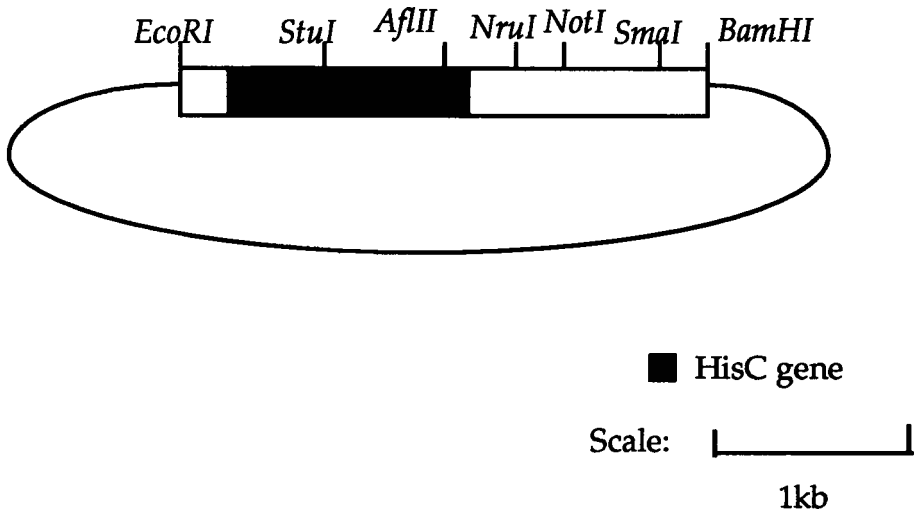


FIG 11: Construction of plasmid pHC and pHC12

Plasmid pHC12 was constructed by cloning the 2.7 kbp *Bam*HI-*Eco*RI fragment from pT4C into the *Bam*HI-*Eco*RI site of pGEM7(-). Site-directed mutagenesis was carried out on pHC to change the 12th codon from TAC to TAG. The resultant plasmid is named pHC12

3.5 Mutagenesis of the *hisC* gene

The first tyrosine triplet in the *hisC* gene occurs at codon position 12. This codon was chosen for mutation to an amber stop codon because termination will occur close to the site of initiation, giving rise to a very short polypeptide product which should be totally non-functional. Furthermore, a tyrosine codon was chosen because suppression with the altered tRNA will give rise to the wild type amino acid sequence. Figure 12 showed the result of site-directed mutagenesis on the plasmid pHC. Oligonucleotide 5 was used to change the first tyrosine codon to an amber codon by changing the C residue at position 36 to a G. The resulting plasmid was named pHC12.

3.6 Strategy for construction of *hisC*(Am) strain

We wished to construct a *hisC*(Am) *H. volcanii* strain by displacing the wild type *hisC* gene from WFD11 with the *hisC*(Am) mutant gene so that the resultant construct would contain only one copy of the gene. Since *H. volcanii* is relatively active in recombination, this will reduce the possibility of restoration of function by recombination between two copies of mutant *hisC* genes.

The scheme for the gene replacement strategy is shown in figure 13. To introduce the *hisC*(Am) gene into the genome, a non-replicating plasmid carrying the gene with a selectable marker was constructed. The 2.7 kbp *Bam*HI *Eco*RI fragment containing the mutated *hisC* gene from plasmid pHC12 was cloned into the *Bam*HI *Eco*RI site of pGOT1 to form pHC12M. The plasmid pGOT1 was constructed by Bissonette (unpublished) by cloning a 2.0kbp DNA fragment which contains the mevinolin resistance gene of *H.volcanii* into the *Xho*I *Asp*718 site at

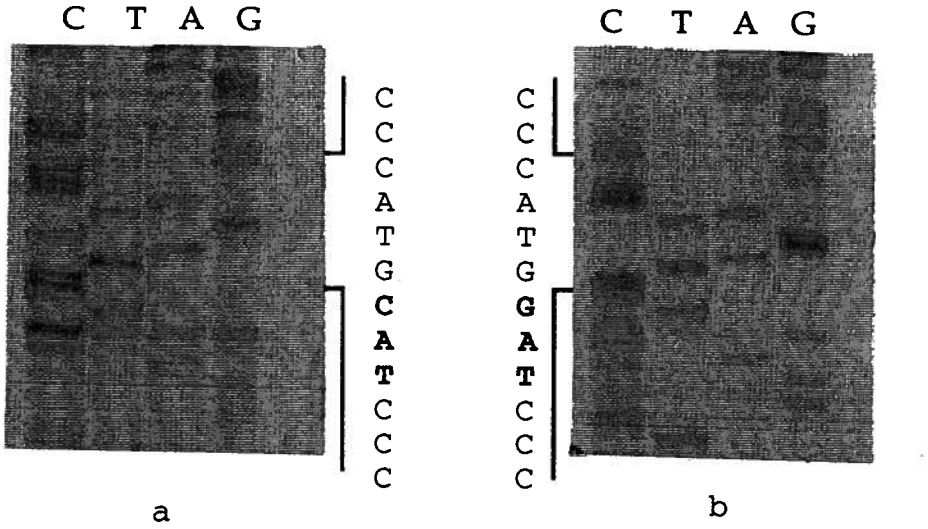


FIG 12: Site-directed mutagenesis of the *hisC* gene

Panel (a) shows the DNA sequence of the wild type *his*Cgene from position 31 to 42. Site-directed mutagenesis was carried out to change the residue C to a G at position 36 so that the twelve codon is changed from a tyrosine to an amber stop codon. Panel (b) shows the DNA sequence of the *hisC*(Am) gene from position 31 to 42 after site-directed mutagenesis. The resultant plasmid is named pHC12.

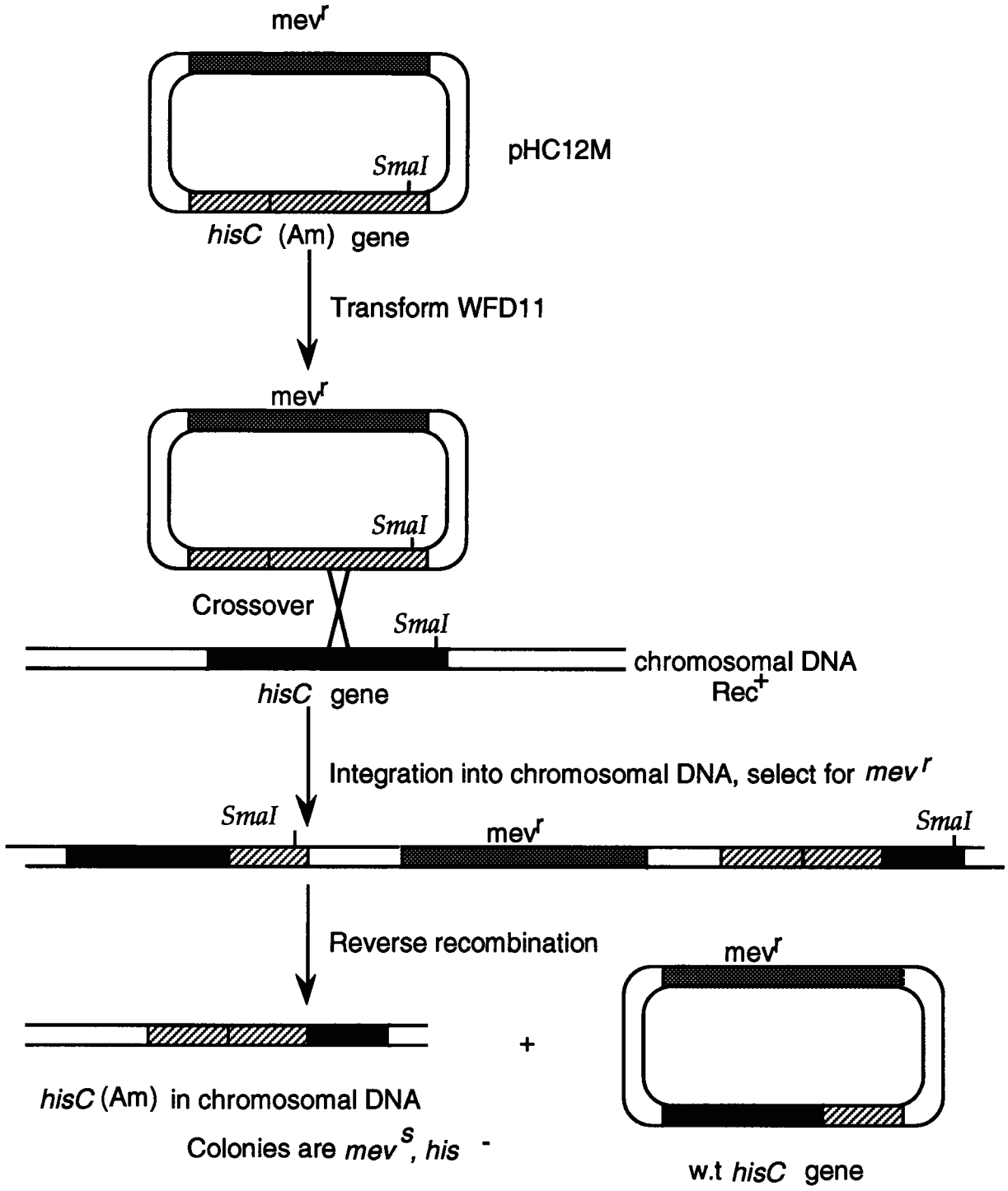


FIG 13: Strategy for constructing the *his* (Am) *H. volcanii* strain

the F1 origin of pGEM3(-). The plasmid pHC12M was then transformed into the wild type *Haloferax volcanii* WFD11 and selected for on mevinolin plates. Since the circular plasmid lacks an origin for replication in *H. volcanii*, it was expected to integrate into the bacterial chromosome by a single crossover event as in figure 2. Transformed cells which are mevinolin resistant will carry two copies of the *hisC* gene and the mevinolin resistance gene in the chromosome. The plasmid can integrate into one of the two loci, the mevinolin gene locus, or the *hisC* gene locus. Ideally, 50% of the time it should integrate into the *hisC* locus, giving rise to a tandem repeat of *hisC* gene flanking the mevinolin resistance gene.

By releasing the antibiotic selection, the cells can undergo reverse recombination (Krebs *et al*, 1993) between the repeated sequences. In the case where the construct has gone into the *mev* locus, one of the *mev* genes (w.t. or mev^r) will be lost together with the *hisC*(Am) gene, giving rise to colonies which are still prototrophic for histidine. Depending on the position of recombination within the repeats, some of the colonies will then lose resistance to mevinolin while the others remain mev^r.

In the other case where the construct goes into the *hisC* gene locus, either copy of the *hisC* gene will be excised together with the mevinolin resistance gene, giving rise to mevinolin sensitive colonies. Half of the colonies will be his^+, when the *hisC*(Am) gene is lost, and the rest his^-. These will be *H. volcanii* strains which have lost the wild type *hisC* gene and now carry only one copy of the gene with an amber mutation in the first tyrosine codon. In other words, the *H. volcanii* *hisC*(Am) strain will be his^- and mev^s.

Two separate attempts were carried out to displace the wild type *hisC* gene

together with the antibiotic resistance gene from the genome by removing the antibiotic selection pressure. In the first trial, the pool of transformed colonies was grown in rich medium until stationary phase was reached (1 week). Ten microlitres of the culture was then inoculated into another 20ml rich medium and grown until stationary phase was reached again. In the second trial, the transformed colonies were grown to stationary phase in rich medium with mevinolin. Then they were transferred to rich media without the antibiotic and grown as above through three more dilutions. The final cultures in both trials were then spread onto rich plates to isolate individual colonies. The colonies were then tested for histidine auxotropy and mevinolin sensitivity. In trial 1, about 10% of the colonies obtained were mevinolin sensitive. In the second trial, about 50% of the colonies were mevinolin sensitive but in both attempts, no histidine auxotrophs were isolated.

3.7 Test for incorporation of the pHC12M construct

To determine whether the non-replicating plasmid pHC12M had actually inserted into the *hisC* locus, Southern hybridization was carried out on genomic DNA digested with *Sma*I obtained from 8 individual mevinolin resistant transformants of WFD11. The *hisC* and *mev* probes used were a 2.5kbp*Sma*I-*Eco*RI fragment from pHC12 (fig. 11), and a 2.0kbp *Asp*718-*Xho*I fragment from pBZL5 (Bissonette and Dennis, unpublished). Figure 14 shows the hybridization pattern of *Sma*I digested genomic DNA of WFD11 and the mevinolin resistant colonies. A novel band of approximately 8 kbp in size which hybridizes to both probes was observed in the transformants. In addition, the *hisC* probe hybridized to a 4.7kbp band in both the wild type and the transformants, whereas a band of around 10 to 11 kbp was observed using the mev probe. All of the 8 colonies examined showed the same hybridization pattern. This result is consistent with integration at the *hisC*

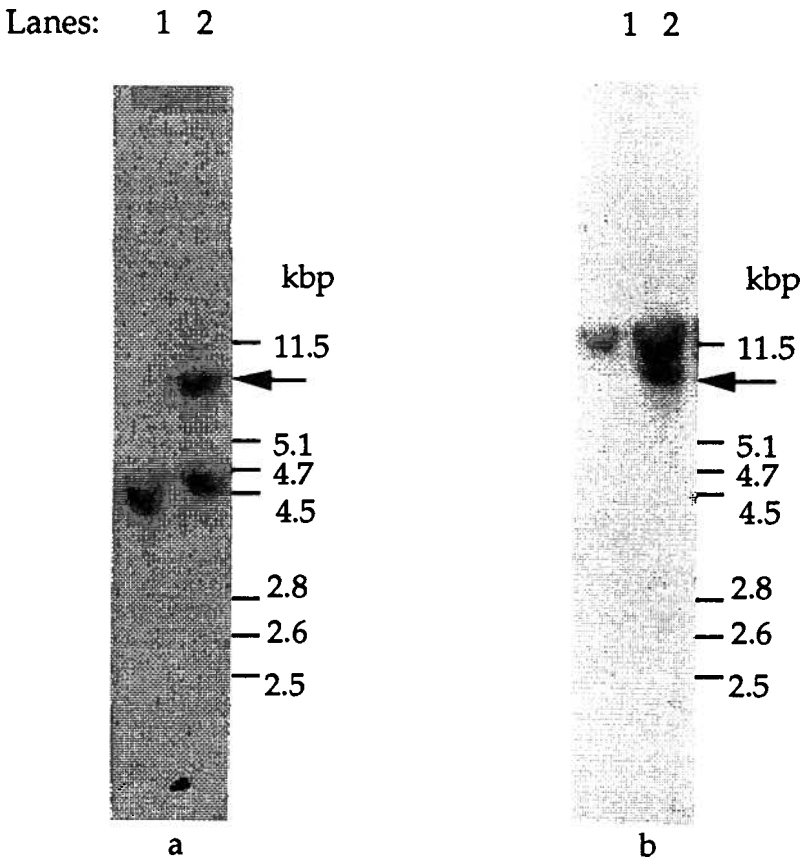


FIG14: Southern hybridization to test for the incorporation of pHC12M into WFD11

Genomic DNA of wild type WFD11 digested with *Sma*I (lane 1) and WFD11 transformants with pHC12M digested with *Sma*I (lane 2) are fractionated by electrophoresis, transferred to nitrocellulose, and probed with $\alpha^{32}P$ labeled restricted fragment containing the *his*C gene (a) or the mevinolin resistance gene (b). In(a), a band of 4.7kbp in size was observed in both lanes, and a band of about 8 kbp as indicated by the arrow in lane 2. In b), a band of 10 to 11 kbp in size is observed in both lanes, and a band of about 8 kbp is observed in lane 2 as indicated by the arrow.

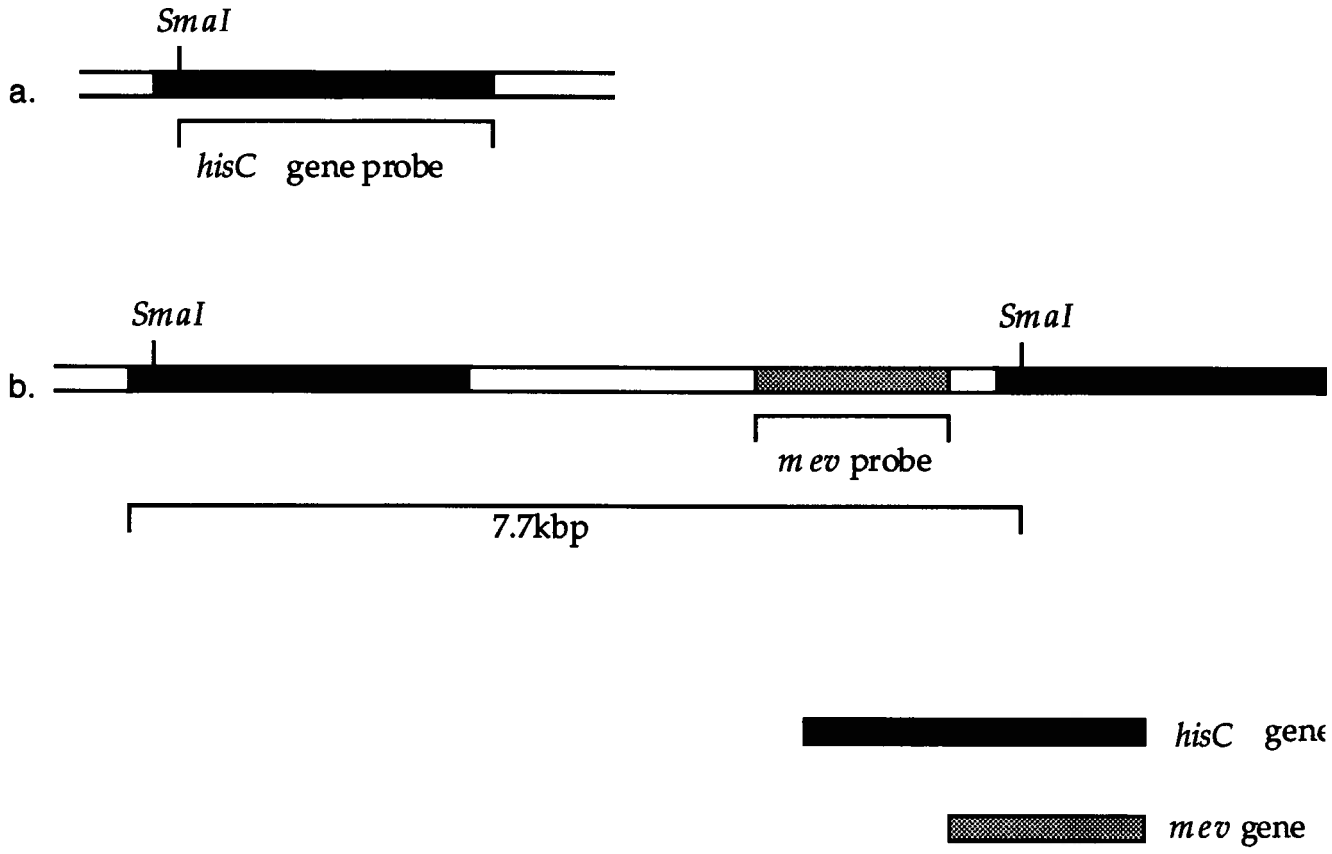


FIG 15: Predicted result of Southern hybridization when the construct is integrated into the *hisC* locus

(a) shows the genomic DNA around the *hisC* locus in the wild type *H. volcanii* WFD11. When the construct pHC12M is correctly integrated into the *hisC* locus, two copies of the gene will be present with a mevinolin resistant gene in between(b). Southern hybridization on *Sma*I digested genomic DNA using *hisC* as probe will give a band of undetermined size in both (a) and (b). An additional band of 7.7kbp(3.0kbp vector + 2.7 kbp *hisC* gene + 2.0 kbp *mev*^{r} gene) as indicated will be observed in (b). This band should also hybridize to the mev probe.

locus (fig 15). However, it seems that none of the colonies had undergone gene displacement by excising the wild type *his*C gene. Therefore, I was unable to construct a *his*C(Am) mutant.

3.8 Test for natural amber suppression

One possible reason why the construction of the *his*C(Am) strain was not successful could be due to the fact that a natural amber suppressor is already present in the wild type genome. Therefore even if the *his*C(Am) gene was introduced, the resultant colonies would still be *his*$^+$. To test this hypothesis, I transformed WR256(*his*$^-$, *arg*$^-$), a derivative of WFD11 that is auxotrophic for histidine and arginine, with plasmid p24H (*his*C(Am) gene in pGOT24). Transformants were selected on rich mevinolin plates. The resultant colonies were streaked individually onto minimal plates with mevinolin and arginine and either with or without histidine to test for auxotrophy. All colonies required histidine for growth. This indicates that all the colonies were still *his*$^-$, therefore there appears to be no natural amber suppressor in *H. volcanii*.

3.9 Test for differential growth rate

In order to confirm that the result obtained in the gene replacement experiments was not due to differential growth of *his*$^+$ or *his*$^-$ strains in the same culture,
WFD11 and WR256 were inoculated into rich media in a ratio of 1:1 in 1000x dilution and grown together for 10 days until stationary phase was reached. Individual colonies from the culture were then tested on minimal plates. Out of 50 colonies tested, 16 were *his*$^+$, leading to the conclusion that there is no significant

difference in the growth rate of the two strains.

3.10 Plasmid construction

Various plasmids were constructed during the course of this project for transformation and for future use. Plasmids that carry the amber suppressor or the *his*C(Am) gene with antibiotic resistance marker (mevinolin or novobiocin) were constructed (table 3). In all cases, the amber suppressor was purified as a 1.5 kbp fragment from plasmid pT3 (fig 8), and the *his*C(Am) gene as a 2.7kbp fragment from pHC12 (fig 11).

Various attempts to construct a plasmid which contains both the amber suppressor and the *his*C(Am) gene were carried out (table 4). The fragments were ligated together overnight and transformed into DH5α. In all cases, no transformants were observed. A control experiment was then carried out with the pHC12-amber
suppressor construct as in table 5, ligations in T4 DNA ligase were carried out in all reactions overnight and then transformed into DH5α. Again, no colonies were observed when the ligation mixture was transformed into DH5α. When transformed into JM101, hundreds of colonies were observed on the plate. However, none of the colonies analyzed contained the 1.5kbp fragment.

3.11 Test for amber suppression in *Haloferax volcanii*

Since the attempts to displace the wild type *his*C gene from WFD11 with *his*C(Am) gene had failed, an alternative to test whether amber suppression can occur is to put the *his*C(Am) gene into the genome of a *his*$^-$ strain and then test for

Plasmid	amber sup.	*hisC*(Am)	*mev*^{r}	*nov*^{r}	*Hvo* ori.	remark
p24H		X	X		X	*Bam*/*Xba*/pGOT25
p24T	X		X		X	*Bam*/*Xba*/pGOT24
pHC12M		X	X			*Bam*/*Eco*/pGOT1
p44H		X		X	X	*Bam*/*Xba*/pGOT44
p44T	X			X	X	*Bam*/*Kpn*/pGOT44
pN7		X		X		*nov*^{r} fragment from pMDS10 (BstYI/NsiI) in pHC12

TABLE 3: Construction of plasmids that carry either the *hisC*(Am) gene or the putative amber suppressor in pGOT vectors
Crosses indicate presence of the gene/fragment.

vector-cut with	amber suppressor-cut with	*hisC*(Am)-cut with
pGOT25-*Eco*RI/*Xba*I	*Bam*HI/*Xba*I	*Sau*3A/*Eco*RI
pGOT44-*Xba*I/*Sph*I	*Bam*HI/*Xba*I	*Sau*3A/*Sph*I
pGEM7-*Eco*RI/*Xba*I	*Bam*HI/*Xba*I	*Sau*3A/*Eco*RI
pHC12-*Sma*I	*Sma*I	

TABLE 4: Attempts to construct a plasmid which contains both the amber suppressor and the *hisC*(Am) gene
In all trials, the vector, *hisC*(Am) gene and the putative amber suppressor were cut with the indicated restriction enzymes. All fragments were then ligated overnight in T4 DNA ligase and transformed into DH5α.

		Colonies
Control 1	pHC12/SmaI	+++
Control 2	pHC12/SmaI dephosphorylated	-
Ligation mix.	pHC12/SmaI dephosphorylated+1.5kb fragment containing the amber suppressor	-

TABLE 5: Control experiment for incorporation of the putative amber suppressor into pHC12

The plasmid pHC12 was cut with *Sma*I, dephosphorylated and then incubated with *Sma*I fragments containing the amber suppressor in T4 DNA ligase. The controls and the ligation mixture were then transformed into DH5α. Hundreds of colonies were observed in control 1. No colony was observed in the other two cases.

restoration of *hisC* gene function by putting in the putative amber suppressor. The plasmid pHC12M (*hisC*(Am) and mevinolin resistance gene with no replication origin) was transformed into WR256 (his^-, arg^-). Transformants were designated WR256.H12. WR256.H12 was then transformed with plasmid p44T (pGOT44 containing the altered tyrosine tRNA gene). Transformants were selected on mevinolin plus novobiocin plates. Individual transformants were then tested for histidine auxotrophy by growing restoration of *hisC* gene function by putting in the putative amber suppressor. The plasmid pHC12M (*hisC*(Am) and mevinolin resistance gene with no replication origin) was transformed into WR256 (his^-, arg^-). Transformants were designated WR256.H12. WR256.H12 was then transformed with plasmid p44T (pGOT44 containing the altered tyrosine tRNA gene). Transformants were selected on mevinolin plus novobiocin plates. Individual transformants were then tested for histidine auxotrophy by growing on minimal plates with arginine . All transformants required histidine for growth.

Southern hybridization was carried out on genomic DNA digested with *Sma*I to confirm the presence of the *hisC*(Am) gene in WR256.H12. Probes used were the *hisC* gene probe and the *mev* probe as in illustrated in figure 15. Using the *hisC* gene probe, a single band of 4.7 kbp was observed in both the wild type DS2 DNA and the WR256.H12. A single band of 10 to 11 kbp was also observed in all lanes when the *mev* gene was used as the probe (fig 17). The result showed that the *hisC*(Am) gene apparently did not integrate into the genome of WR256.H12, and only one copy of the *mev* gene was present. Therefore no conclusion about amber suppression in *H. volcanii* can be drawn yet.

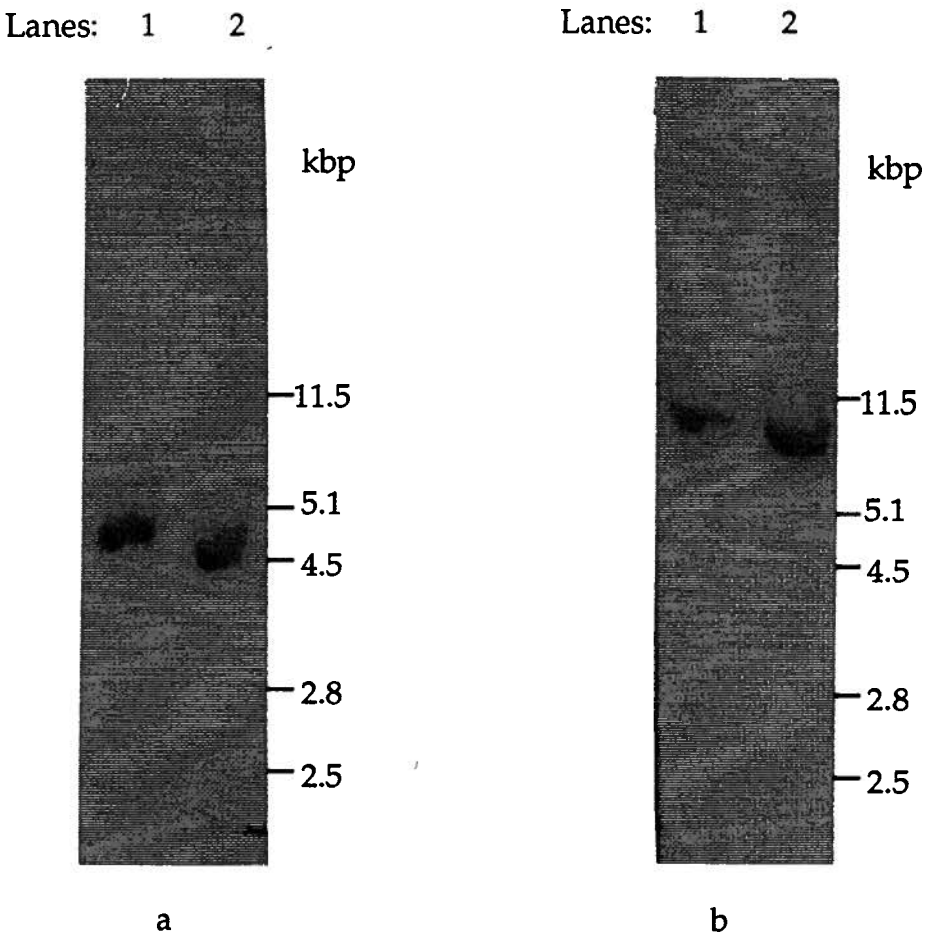


FIG 16: Southern hybridization to test for the incorporation of pHC12M into WR256

Genomic DNA of wild type WFD11 digested with *Sma*I (lane 1) and WR256 transformants with pHC12M digested with *Sma*I (lane 2) are fractionated by electrophoresis, transferred to nitrocellulose, and probed with ^{32}P labeled restricted fragment containing the *his*C gene (a) or the mevinolin resistant gene (b). In(a), a band of 4.7kbp in size was observed in both lanes. In (b), a band of 10 to 11 kbp in size is observed in both lanes.

DISCUSSION

4.1 THE TYROSINE TRANSFER RNA

The presence of the *H.volcanii* tyr tRNA gene was detected by Southern hybridization. It is present on the *Sma*I genomic digest as a 1.5 kbp fragment. WFD11 and DS2 are essentially the same, except that WFD11 lacks the plasmid pHV2. Southern hybridization indicates that the location of the tyrosine tRNA gene is identical in both genomes.

A size-fractionated *Haloferax volcanii* genomic library constructed by cloning the 1.4 to 1.6kbp genomic *Sma*I fragments was constructed. The recognition sequence of the restriction enzyme *Sma*I is CCCGGG, and the enzyme cuts between the C and G to give blunt ends. The clone containing the plasmid pT1 obtained by screening the library with oligonucleotide 1 and 2 was confirmed to contain the tyrosine tRNA gene by Southern hybridization. However, restriction mapping showed an insert of 4.5kbp in size. The plasmid pT1 is shown in fig. 6. Since blunt end ligation was carried out, three 1.5kbp fragments were cloned into the dephosphorylated *Sma*I site of pGEM7(-) and the first *Sma*I site is lost during the religation process. The fragment which contains the tyrosine tRNA gene lies in the middle fragment. An *Eco*RI site is present at 0.6kbp from the first *Sma*I site, and is 38 bp upstream of the tyrosine tRNA coding sequence. The DNA sequence of this region is shown in fig 7. The gene is 74 bp long and agrees with the RNA sequence of the tyrosine transfer RNA (fig 3). Putative upstream promoter elements were identified, which show a high sequence homology as compared with the consensus sequence of upstream promoter regions of various other *H. volcanii* tRNA genes(fig 17) (Daniels *et al*, 1986). In the *H. volcanii* tyrosine tRNA gene, the upstream

Upstream region of tyrosine tRNA:

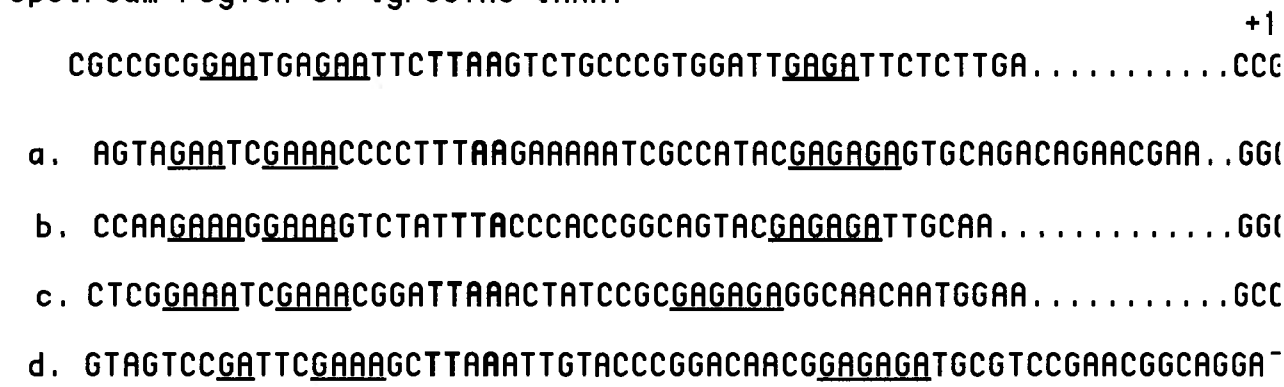


Fig 17: Comparison of halobacterial tRNA 5′-flanking region

The upstream promoter region of tyrosine tRNA gene is compared with various other tRNA genes already cloned: a. Trp; b. Lys; c.Ser; d. Val. (adapted from Daniels et al., 1986). The TTAA putative promoter is identified and shown in bold letters. Other 5′-flanking consensus sequences are observed as GA rich regions (underlined). The first three nucleotides of the tRNA are also shown. The conserved sequence blocks GAA, GAA, TTAA and GAGAGA are observed in the upstream region nof all the tRNA examined here. Although the distances separating these blocks are variable, this conservation in sequence is remarkable.

elements GAA is present at positions -44 and -38, GAGA at position -13; the TTAA box is also observed at position -32, and is believed to be homologous to the TATA box in eukaroytes (Hausner *et al.*, 1991; Reiter *et al*, 1990; Shimmin and Dennis, 1989; Thomm and Wich, 1988). The promoter region has a very high A+T content compared to the rest of the genome. RNA polymerase finds and binds to the promoter region. It unwinds the DNA helix to allow initiation of translation of the tRNAs. Since the A+T base pairs at the TTAA region are comparatively weaker than G+C base pairs, the region is more susceptible to "melting", or formation of an open complex.

The end of the gene is marked by a poly(T) tract at position +75. It consists of a stretch of 4 Ts, which is believed to be the termination sequence in archaebacteria (Shimmin and Dennis, 1989). Shimmin and Dennis (1989) showed by S1 nuclease protection that the 3′ transcript end site of RNAs are located within runs of T residues and are often preceded by GC rich sequences. Long T clusters adjacent to yeast tRNA had been shown to efficiently terminate transcription by RNA polymerase III (Daniels and Hall, 1985). Since an RNA-DNA hybrid consisting of polyribo U and polydeoxy A is very unstable, the RNA chain will be expelled from the DNA duplex leading to termination of transcription. Since poly Ts are involved in termination, there is a strong preference to avoid codon with adjacent T residues and the TTT codon for phenylalanine is utilized sparingly in *H.volcanii*. On the other hand, in both non-coding regions and on the minus strand of coding regions, T runs are more prevalent.

4.2 MODIFICATION PATTERN

Modified bases are very common in archaebacterial tRNAs. In the *H. volcanii*

tRNA, an unidentified nucleoside was observed from RNA sequencing at position 15. This is not found in any eubacterial or eukaryotic tRNAs. DNA sequencing of the cloned gene indicates that it comes from a guanosine. Gupta and Woese (1980) proposed that it is a modified G whose pKa is considerably higher than that of an unmodified G. It could be a 2-amino, 6-oxypyrimidine, a pseudo-cytodine, a 2-aminopurine or some hypermodified base containing an exo-cyclic amino group at the 2-carbon position of the ring. The function of this modification is not known.

An inosine can be formed by removing the NH_2 group from a G or an A. In the tyrosine tRNA, an A was present at the corresponding position in the DNA sequence. Since modified nucleosides are constantly encountered in tRNAs of different organisms, Nishimura (1979) suggests that they play an important role in tRNA functions, but no real proof has been discovered yet.

As in the case with eukaryotic tRNAs, the CCA terminal is added to the tRNA molecule post-transcriptionally. Transfer RNA lacking the CCA end has been found in *B. subtilis* and the archaebactium *Sulfolobus solfataricus* (Vold, 1985). The 3' terminal adenosine can become attached to the corresponding amino acid by covalently bonding with the carboxyl group of the amino acid. This reaction is catalyzed by aminoacyl-tRNA synthetase.

4.3 THE *his*C GENE

The *hisC* gene encodes histidinol phosphate aminotransferase (Conover and Doolittle, 1990), which is the eighth enzyme of the histidine biosynthetic pathway. It catalyzes the conversion of imidazole acetol phosphate to L-histidinol phosphate (Watsons *et al*, 1987).

The first tyrosine codon was chosen to be mutated to the amber stop codon so that only a very short polypeptide will be transcribed. This reduces the possibility of forming a partially functional polypeptide product. The DNA sequence of the region is changed from TAC to TAG, so that on the mRNA the corresponding tyrosine codon was changed from UAC to UAG. Theoretically, it can now be recognized by the anticodon CUA of the putative amber suppressor.

4.4 GENE REPLACEMENT EXPERIMENTS

Two individual trials of the gene replacement experiment were carried out. In trial 1, only 10% of reverse recombination in the mev gene was observed. Another trial was then carried out where the process was carried out for a longer period of time over more generations to get a 50% mev reverse recombination rate. In both cases, 100 colonies were screened. However, no *his*$^-$ colony was observed. This could be due to:

1) A natural amber suppressor might be already present in *H. volcanii* so that colonies that did undergo gene replacement were still *his*$^+$ since the *his*C(Am) gene is suppressed.

2) There is a significant difference in growth rate between *his*$^+$ and *his*$^-$ strains. During the process of growing the two strains together in rich media, the *his*$^+$ strains might outgrow the *his*$^-$ strains. This effect might be able to eliminate all the *his*$^-$ strains since they were grown together for more than 20 generations.

3) The *his*C(Am) gene is polar. The *his*C(Am) gene may affect the expression

of any genes downstream of it so that displacement of the wild type gene to leave one copy of the *his*C(Am) gene is unfavorable.

In order to test whether a natural amber suppressor is already present in the system, the plasmid p24H (*his*C(Am) gene in pGOT24) was transformed into WR256 (*his*$^-$, *arg*$^-$) and transformants were selected on mevinolin plates. WR256 is a *his*$^-$ strain in which the *his*C gene is randomly mutated. If an amber suppressor is already present, this *his*C(Am) gene will be suppressed, giving rise to *his*$^+$ colonies. All colonies still required histidine for growth, leading to the conclusion that no natural amber suppressor is present in *H. volcanii*.

The test for differential growth rate of *his*$^+$ and *his*$^-$ strains indicates that when the two are grown together in rich media for a period of time, the ratio of them remains constant. Therefore the second reason is not correct since the *his*$^+$ strains will not be able to outgrow the *his*$^-$ strains in the same media.

However, the 50% loss in mevinolin resistance indicated that reverse recombination did take place. The result from the Southern hybridization shows that the construct did go into the *his*C gene locus. However, displacement of the wild type *his*C gene did not occur. Since the gene replacement procedures were carried out in rich media, selection on histidine prototrophs should not exist. Another Southern hybridization for the genomic DNA of the colonies after the gene replacement experiments would indicate whether one or both copies of the *his*C gene was present and might give a better insight on why the experiment was not successful.

4.5 PLASMID CONSTRUCTION

Plasmids carrying the putative amber suppressor or the *hisC*(Am) gene with mevinolin resistance or novobiocin resistance and with or without replicative origin in *H. volcanii* were constructed for further use (table 3).

We attempted to construct a plasmid which carries both the amber suppressor and the *hisC*(Am) gene. By transforming the plasmid into the *his*$^-$ strain WR256, we hope to test whether the modified tRNA could suppress the *hisC*(Am) gene (table 4). However, we were unable to put the two genes together on the same plasmid. The reason for this failure is unclear. The genes on the plasmid pGEM7(-) should not be expressed by *E.coli*. We could not explain the fact that the ligation mixture pHC12+amber suppressor fragment transformed JM101 but not DH5α. Transformed plasmids obtained from JM101 transformants were analyzed and no 1.5kbp *Sma*I fragment was observed. However, we were able to recover the 2.7 kbp *hisC* gene fragment by cutting with *Bam*HI and *Eco*RI, although the cutting efficiency was much lower than with the original clone, indicating that some rearrangement might have occurred.

4.6 TEST FOR AMBER SUPPRESSION IN *HALOFERAX VOLCANII*

Southern hybridization indicates that the pHC12M construct did not recombine into the WR256 genome since only one copy of the *hisC* gene was present. Furthermore, only one copy of the *mev* gene was present. Since the transformants were selected on mevinolin plates, the mevinolin resistance gene must have replaced the wild type *mev* gene in WR256 by two crossover events (fig 2). According to Mevarech (personal communication), the mevinolin gene has a high tendency to recombine into the genome to give mevinolin resistant colonies.

However, the fact that pHC12M was successfully integrated into WFD11 in all analyzed colonies in the attempt to construct a *his*C(Am)*H. volcanii* strain indicates that this approach is feasible. The problem might lie in the strain WR256 since it was mutagenised randomly, and no further study on the strain is available. The other antibiotic resistance gene, novobiocin, could be used in place of the mevinolin resistance gene to repeat the above experiment. In this case, the resultant *nov*^{r} colonies should be transformed again with a plasmid that carries the *mev*^{r} marker and the putative amber suppressor. The double transformants should then be tested for histidine auxotrophy, and hence the ability for amber suppression to occur in *Haloferax volcanii*.

4.7 CONCLUSION

Since not much is known about the *H. volcanii* system, many difficulties were encountered during the course of the project. Some further experiments, however, are possible:

1. Gene replacement experiment: Southern hybridization to analyze the genome after the reverse recombination step. This might give further insight as to why the displacement of the wild type *his*C gene was not successful.

2. Test for amber suppression: The experiment can be repeated using novobiocin instead of mevinolin as the antibiotic selection marker in the first transformation since it has less tendency to recombine into the genome to form novobiocin resistant colonies. However, novobiocin transformants take more than two weeks to be visible and transformation efficiency is much lower than with the

mevinolin vectors. The mev vectors can then be used to put the putative amber suppressor into the transformants. Since the mev vector in the second transformation contains a *H. volcanii* origin, recombination of the mev gene to form *mev*^{r} colonies should be minimized.

During this project, the tyrosine tRNA gene of *H. volcanii* was cloned and sequenced. A putative amber suppressor and a *hisC* gene that carries an amber stop codon were constructed. Although the attempts to construct a *hisC*(Am) *H. volcanii* mutant failed and the test for amber suppression was inconclusive, the established products and procedures of this project should provide the basic foundation for any further investigation of amber suppression in *H. volcanii.*

REFERENCES

Abelson, J.N., Gefter, M. L., Barnett, L., Landy, A. Russell, R.L. and Smith, J.D. (1970). Mutant tyrosine transfer ribonucleic acids. J. Mol. Biol. 47, 15-28

Adams, J.M. and Capecchi, M.R. (1966). N-formylmethionyl-sRNA as the initator of protein synthesis. Proc. Natl. Acad. Sci. USA 55,147-155

Allison, D. S. and Hall, B. D. (1985). Effects of alternations in the 3′ flanking sequence on *in vivo* and *in vitro* expression of the yeast SUP4-o $tRNA^{Tyr}$ gene. EMBO. 4, 2657-2664

Boch. A and Kandler,O. (1985). Antibiotic sensitivity of archaebacteria, p525-544. *In* Woese, O.R. and Wolfe, R.S. (ed), The Bacteria-a treatise on structure and function, vol 8, The Archaebacteria, Academic Press, N.Y.

Cabrera, J. A., Bolds, J., Shields, P.E., Havel, C.M. and Watsons, J. A. (1985). Isoprenoid synthesis in *Halobacterium halobium*. J. Biol. Chem. 261, 3578-3583

Charlebois, R. L., Lam, W. L., Cline, S. W. and Doolittle, W. F. (1987). Characterization of pHV2 from *Halobacterium volcanii* and its use in demonstrating transformation of an archaebacterium. Proc. Natl. Acad. Sci. 84,8530-8534

Charlebois, R. L., Schalkwyk, L.C., Hofman, J.D. and Doolittle, W.D. (1991). Detailed physical map and set of overlapping clones covering the genome of the archaebacterium *Haloferax volcanii* DS2. J. Mol. Biol. 222,509-524

Chowdhury, K. (1991). One step 'miniprep' method for the isolation of plasmid DNA. Nucleic Acid Res. 19, 2792

Cline, S. W. and Doolittle, W.F. (1987). Efficient transfection of the archaebacterium *Halobacterium halobium*. J. Bacteriol. 169, 1341-1344

Cline, S., Schalkwyk, L.C. and Doolittle, W.F. (1989). Transformation of the

archaebacterium *Halobacterium volcanii* with genomic DNA. J. Bacteriol. 171, 4987-4991

Cohen, A., Lam, W. L., Charlebois, R.L., Doolittle, W. F. and Schalkwyk, L. C. (1992). Localizing genes on the map of the genome of *Haloferax volcanii*, one of the Archae. Proc. Natl. Acad. Sci. 89, 1602-1606

Crick, F. (1966). The genetic code. Cold Spring Harbor Symposia on Quantitative Biology. vol 8. The Genetic Code. Cold Spring Harbor, L.I., N.Y.

Daniels, L.L. and Wais, A.C. (1984). Restriction and modification of halophage S45 in *Halobacterium*. Curr. Microbiol. 10, 133-136

Dennis, P.P. (1986). Molecular biology of archaebacteria. J. Bacteriol. 168, 471-478

Dennis, P.P. (1993). The molecular biology of halophilic archaebacteria,p255-288. *In* Vreeland, R.H. and Hochstein, L.I. (ed), The Biology of Halophilic Bacteria, CRC Press.

Doolittle, W.F. (1985). Genome structure in archaebacteria, p545-564. *In* Woese, O.R. and Wolfe, R.S. (ed), The Bacteria-a treatise on structure and function, vol 8, The Archaebacteria, Academic Press, N.Y.

Eikmanns, B. and Thauer, R.K. (1984). Catalysis of an isotopic exchange between CO_2 and the carboxyl group of acetate by *Methanosarcina barkeri* grown on acetate. Arch. Microbiol. 138, 365-370

Engelhardt, D.L., Webster, R.E., Wilhelm. R.C. and Zinder, N.D. (1965). In vitro studies on the mechanism of suppression of a nonsense mutation. Proc. Natl. Acad. Sci. 54, 1791-1797

Garen, A. (1968). Sense and nonsense in the genetic code. Science 160,149-159

Gesteland, R.F., Salser, W. and Bolle, A. (1967). In vitro synthesis of T4 lysozymes by suppression of amber mutations. Proc. Natl. Acad. Sci. 58,2036-2042

Goodman, H.M., Abelson J., Landy, A. Brenner, S. and Smith, J.D. (1968). Amber suppression: a nucleotide change in the anticodon of a tyrosine transfer RNA. Nature. 217, 1019-1024

Gupta, R. (1984). *Halobacterium volcanii* tRNAs. J. Biol. Chem. 259, 9461-9471

Gupta, R. (1985). Transfer ribonucleic acids of archaebacteria. p311-343. *In* Woese, O.R. and Wolfe, R.S. (ed), The Bacteria-a treatise on structure and function, vol 8, The Archaebacteria, Academic Press, N.Y.

Gupta, R. and Woese, C.R. (1980). Unusual modification patterns in the transfer ribonucleic acids of archaebacteria. Curr. Microbiol. 4, 245-249

Hausner, W., Gerhard, F. and Thomm, M. (1991). Control regions of an archael gene, a TATA box and an initiator element promote cell-free transcription of the $tRNA^{Val}$ gene of *Methanococcus vannielii*. J. Mol. Biol. 222, 495-508

Hill, C. W. (1975). Informational suppression of missense mutation. Cell. 6,419-427

Hirsh, D. (1971). Tryptophan transfer RNA as the UGA suppressor. J. Mol. Biol. 58, 439-458

Hirsh, D. and Gold, L. (1971). Translation of the UGA triplet *in vivo* by tryptophan transfer RNA's. J. Mol. Biol. 58, 459-468

Hoagland, M.B., Stephenson, M.L., Scott, J.F., HEcht, L.I. and Zamecnik, P.C. (1957). A soluble ribonucleic acid intermediate in protein synthesis. J. Biol. Chem. 231, 241-257

Holley, R. W., Apgaz, J., Everett, G. A., Madison J.T., Marquisee, M., Merril, S.H., Penswich, J. R. and Zamir, A. (1965). Structure of a ribonucleic acid. Science 147:1462

Holmes, M. L. and Dyall-Smith, M. L. (1991). Mutations in DNA gyrase result in novobiocin resistant in halophilic archaebacteria, J. Bacteriol. 173, 642-648

Holmes, M.L., Nuttall, S.D. and Dyall-Smith, M.L. (1991). Construction and use of Halobacterial shuttle vectors and further studies on *Haloferax* DNA gyrase. J. Bacteriol. 173, 3807-3813

Hui, I. and Dennis. P (1985). Characteristic of the ribosomal RNA gene clusters in *Halobacterium cutirubrum.* J. Biol. Chem. 260,899-906

Kates, M., Wassef, M.K., and Kushner, D.J. (1968). Radioisotopic studies on the biosynthesis of the glyceryl diether lipids of Halobacterium cutirubrum. Can. J. Biochem. 46,971-977

Kleckner, N. (1981). Transposable elements in prokaryotes. Ann. Rev. Genet. 15,341-404

Kondo, S., Yamagishi, A. and Oshima, T. (1991). Positive selection of the uracil auxotrophs of the sulfur-dependent thermophilic archaebacterium *Sulfolobus acidocaldarius* by use of 5-fluoroorotic acid. J. Bacteriol. 173, 7698-7700

Krebs, M.P., Mollaaghababa, R. and Khorana, A.G. (1993). Gene replacement in *Halobacterium halobium* and expression of bacteriorhodopsin mutants. Proc. Natl. Acad. Sci. 90, 1987-1991

Kunkel, T.A. (1985). Rapid and efficient site-specific mutagensis without phenotypic selection. Proc. Natl. Acad. Sci. 82, 488-492

Kunkel, T.A., Roberts, J.D. and Zakour, R.A. (1987). Rapid and efficient site-specific mutagensis without phenotypic selection. Meth. Enzymol. 154,367-382

Lam, W. L. and Doolittle, W. F. (1989). Shuttle vectors for the archaebacterium *Halobacterium volcanii.* Proc. Natl. Acad. Sci. 86, 5478-5482

Lewin, B. (1974). Gene expression, vol 1, Bacterial genomes, William Clowes & Sons Limited, London

Mevarech, M. and Werczberger, R. (1985). Genetic transfer in *Halobacterium*

volcanii. J. Bacteriol. 162, 461-462

Mizuuchi, K., O'Dea, M. H. and Gellert, M. (1978). DNA gyrase: subunit structure and ATPase activity of the purified enzyme. Proc. Natl. Acad. Sci. 75, 5960-5963

Moore, R.L. and McCarthy, B.J. (1969a). Characterization of the deoxyribonucleic acid of various strains of halophilic bacteria. J. Bacteriol. 99, 248-254

Moore, R.L. and McCarthy, B.J. (1969b). Base sequence homology and renaturation studies of the deoxyribonucleic acid of extremely halophilic bacteria. J. Bacteriol. 99, 255-262

Mullakhanbhai, M.F. and Larsen, H. (1975). *Halobacterium volcanii* spec. nov., a dead sea halobacterium with a moderate salt requirement. Arch. Microbiol. 104, 207-214

Muriana, F. J. G., Sanchez, M.C., Rodulfo, J. D., Alvarez-Ossorio, M. C. and Relimpio, A. M. (1987). Optimization of the cell envelope of extremely halophilic bacteria. J. Biochem. Biophys. Meth. 14,19-28

Ner, S.S., Goodin, D.B. and Smith, M. (1988). Laboratory methods: a simple and efficient procedure for generating random point mutations and for codon replacements using mixed oligonucleotides. DNA 7,127-134

Nishimura, S. (1979). Modified nucleosides in tRNA, p59-79. *In* Schimmel, P.R., Soll, D. and Abelson, J.N. (ed),Transfer RNA: Structure, Properties, and Recognition, Cold Spring Harbor Laboratory

Reiter, W., Hudepohl, U. and Zillig, W. (1990). Mutationalanalysis of an archaebacterial promoter: essential role of a TATA box for transcription efficiency and start-site selection *in vitro*. Proc. Natl. Acad. Sci. 87, 9509-9513

Rich, A. and RajBhandary, U.L. (1976). Transfer RNA: Molecular structure, sequence, and properties. Annu. Rev. Biochem. 45,805-860

Rosenshine and Mevarech, unpublished result cited in Dennis (1993). The molecular biology of halophilic archaebacteria,p255-288. *In* Vreeland, R.H. and Hochstein, L.I. (ed), The Biology of Halophilic Bacteria, CRC Press.

Russell, R. L., Abelson, J. N., Landy, A., Gefter, M. L., Brenner, S. and Smith, J. D. (1969). Duplicate genes for tyrosine transfer RNA in *Escherichia Coli.* J. Mol. Biol. 47, 1-13

Saks, M. E., Sampsons, J. R. and Abelson, J. N. (1994). The transfer RNA identity problem: a search for rules. Science 263, 191-197

Sambrook, J., Maniatis, T. and Fritsch, E. (1982). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Saunders, S. and Burke, J.F. (1990). Rapid isolation of miniprep DNA for double strand sequencing. Nucleic Acids Res. 18, 4948

Sawadogo, M. and Dyke, M.W.V. (1991). A rapid method for the purification of deprotected oligonucleotides. Nucleic Acids Res. 19, 674

Shimmin, L. C. and Dennis, P. (1989). Characterization of the L11, L1, L10 and L12 equivalent ribosomal protein gene cluster of the halophilic archaebacterium *Halobacterium cutirubrum.* EMBO J. 8, 1225-1235

Singer, B. and Kroger, M. (1979). Participation of modified nucleosides in translation and transcription. Prog. Nucleic Acid Res. Mol. Biol. 23, 151-194

Smith, J.D., Abelson, J. N., Clark, B.F. C., Goodman, H. M. and Brenner, S. (1966). Studies on amber suppressor tRNA, p479-485. *In* Cold Spring Harbor Symposia on Quantitative Biology. vol 8. The Genetic Code. Cold Spring Harbor, L.I., N.Y.

Smith, J.D., Barnett, L. Brenner, S. and Russell, R.L. (1970). More mutant tyrosine transfer ribonucleic acids. J. Mol. Biol. 54, 1-14

Smith,M. (1986). Site Directed Mutagensis. Phil. Trans. R. Soc. Lond. A317,295-304

Spratt, B.G., Hedge, P.J., te Heesen, S., Edelman, A. and Broome-Smith, K. (1986). Kanamycin-resistant vactors that are analogues of plasmids pUC8, pUC9, pEMBL8 and pEMBL9. Gene, 41,337-342

Stretton, A.O.W. and Brenner, S. (1965). Molecular consequences of the amber mutation and its suppression. J. Mol. Biol. 12,456-465

Thiara, A.S. and Cundliffe, E. (1988). Cloning and characterization of a DNA gyrase B gene from *Streptomyces sphaeroides* that confers resistance to novobiocin. EMBO J. 7, 2255-2259

Thomm, M. and Wich, G. (1987). An archaebacterial promoter lelment for stable RNA genes with homology to the TATA box of higher eukaryotes. Nucleic Acids Res. 16,151-163

Thomm,M., Wich, G., Brown, J.W., Frey, G., Sherf,B. and Beckler, G.S. (1989). An archaebacterial promoter sequence assigned by RNA polymerase binding experiments. Can. J. Microbiol. 35,30-35

Watson, J.D., Hopkins, N.H., Roberts, J.W., Steitz, J.A. and Weiner, A.M. (1987). Molecular Biology of the Gene, 4th edition. The Benjamin/Cummings Publishing Company, Inc., Califonia.

Wich, G., Jarsch, M. and Bock, A. (1984) Apparent operon for a 5S ribosomal RNA gene and for tRNA genes in the archaebacterium *Methanococcus vannielii.* Mol. Gen. Genet. 196, 146-151

Woese. C. R. (1987) Bacterial evolution. Microbiol. Rev. 51, 221-271

Woese, C.R. (1981). Archaebacteria. Sci. Amer. 244, 98-122

Woese, C.R., Gutell, R., Gupta, R. and Noller, H.F. (1983). Detailed analysis of the higher-order structure of 16S-like ribosomal ribonucleic acids. Microbiol. Rev. 47, 621-669

Woose, C. R., Kandler O. and Wheelis, M.L. (1990). Towards a natural system of organisms: Proposal for the domains Archaea, Bacteria and Eucarya. Proc. Natl. Acad. Sci. 87, 4576-4579

Zillig, W., Stetter, O., Schnabel, R. and Thomm, M. (1985). DNA-dependent RNA polymerase of the archaebacteria, p499-524. *In* Woese, O.R. and Wolfe, R.S. (ed), The Bacteria-a treatise on structure and function, vol 8, The Archaebacteria, Academic Press, N.Y.