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"Isolation and characterization of motility-defective mutants of *Haloferax volcanii*"

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In the name of Allah,
The most Merciful,
The most Compassionate.

To Mrs. Maryam Farsi J., my mother and
Mrs. Jila Valadkhani, my wife,
Who loved me the way I was and
never stopped believing in me.

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Abstract

Classification of organisms into the three domains archaebacteria, eubacteria and eucaryotes has provided new avenues in understanding evolution and its mechanisms. *Haloferax volcanii* is a motile extreme halophile, and a detailed physical map of its genome exists. An effort is underway to locate more genes and to increase the detail of this map.

Flagellated motile bacteria possess a number of filaments that function as cellular propellers. Motile bacteria swim outward in a low concentration agar medium (swarm medium) and form a swarm. Over sixty genes are involved in motility and chemotaxis. Cloning motility and chemotaxis genes from halophilic archaebacteria would provide interesting comparative data.

I have isolated twenty-five independent motility-defective mutants and four independent super-motile mutants of *Hf. volcanii* WFD11. Some of these mutants were characterized by light and electron microscopy. The motility-defective mutants form three characteristic kinds of swarms on swarm medium: non-motile colonies, small fuzzy colonies and variable-size-colonies. Wild-type *Hf. volcanii* DS2 possesses 4-10 flagellar filaments which form a bundle. In stationary phase culture, some of these peritrichously flagellated cells become elongated and polarly flagellated. Light and electron microscopy of two mutants revealed that these cells that form non-motile colonies are non-motile,

two mutants revealed that these cells that form non-motile colonies are non-motile, although they possess normal looking flagellar filaments. These mutants may have paralyzed filaments. Revertants of these mutants may produce dotted and/or scalloped-edge swarms. Mutants that form small fuzzy colonies were found to be cell cycle defective mutants which were not able to undergo normal cell division and thus continued growing until they formed very long spaghetti-like cells. These *Hf. volcanii* mutants have normal flagellar filaments and are motile when they are short but as they grow longer, they become less and less motile. The third group of mutants with variable size colonies, were normal looking motile cells under the light microscope. Super-motile mutants possess tens of flagellar filaments.

Three independent *Hf. volcanii* DS2 genomic shot-gun libraries were constructed and amplified. These shot-gun libraries are presently being used to genetically complement *Hf. volcanii* mutants by a PEG-mediated transformation procedure. With this progress, now we are ready to clone and map genes involved in motility and chemotaxis and also genes involved in cell cycle and cell division in *Hf. volcanii*.

Résumé

La compréhension de l'évolution, et ses mécanismes, a pris un nouvel envol depuis la classification des organismes en trois domaines, archaebactérie, eubactérie et eucaryotes. *Haloferax volcanii* est un halophile extrème pouvant se mouvoir. Une cartographie physique détaillée de son génome existe. Une étude est présentement en cours afin de localiser plus de gènes et d'augmenter la précision de cette cartographie.

Les bactéries se bougent à l'aide de flagelles possèdant un nombre de filaments jouant rôle de propulseurs cellulaires. Les bactéries mobiles nagent vers l'extérieur sur un médium d'agar de faible concentration et forme une masse. Plus de soixante gènes sont impliqués dans la mobilité et la chémotaxie. Le clonage de gènes impliqués dans ces mécanismes chez une archaebactérie halophile peut fournir des données intéressantes à comparer.

J'ai isolé vingt-cinq différents mutants pour la mobilité et quatre différents mutants ayant une mobilité accrue chez *Hf. volcanii* WFD11. Quelques uns de ces mutants ont été caractérisés par microscopie optique et à électrons. Trois type caractéristiques de masses sont observés sur un médium d'agar de faible concentration chez les mutants non mobiles: colonies non mobiles, petites colonies floues et des colonies de différentes tailles. *Hf. volcanii* DS2 sauvage possède entre 4-10 filaments flagellaires formant un paquet. En culture à phase stationaire, quelques unes de ces cellules possèdant des flagelles

distribuées au hasard deviennent allongées et flagellées de façon polaire.

La microscopie optique et à électrons de deux mutants a révélé que les cellules composant les colonies non mobiles étaient non mobiles malgré la présence de filaments flagellaires apparaissant normaux. Les filaments pourraient toutefois être paralysés.

Les révertants de ces mutants peuvent produire des colonies ponctuelles et/ou des colonies à bordures inégales. Les mutants formant des petites colonies floues ont un cycle cellulaire défectueux: ils sont incapables d'avoir une division cellulaire normale et ils continuent à grandir jusque à ce qu'ils forment de très longues cellules ressemblant à des spaghetti. Ces mutants de *Hf. volcanii* ont des filaments flagellaires normaux et sont mobiles lorsqu'ils sont petits mais au fur et à mesure qu'ils grandissent, ils perdent leur mobilité. Le troixième type de mutants formant des colonies de différentes tailles avait une apparence normale lorsqu'observé au microscope optique. Les mutants ayant une mobilité accrue possèdent des dixaines de filaments flagellaires.

Trois différentes librairies aléatoires de *Hf. volcanii* DS2 furent construites et amplifiées. Elles sont présentement utilisées afin de complémenter génétiquement les mutants de *Hf. volcanii* par une procédure de transformation médiée par le PEG. Grâce à cet avancement, nous sommes maintenant prêts à cloner et à cartographier des gènes impliqués dans la mobilité et la chémotaxie et aussi des gènes impliqués dans le cycle et la division cellulaire chez *Hf. volcanii*.

Abbreviations

A	adenosine
bp	base pair
BR	bacteriorhodopsin
C	cytosine
CCW	counterclockwise
CW	clockwise
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid, disodium salt
EF-G	elongation factor G
EF-Tu	elongation factor Tu
G	guanosine
HR	halorhodopsin
IS	insertion sequence
kbp	kilobase pair
kDa	kiloDalton
Mbp	mega base pair
msec	millisecond
MCP	Methyl-accepting chemotaxis protein
mev	mevinolin
Mr	molecular weight

mRNA	messenger ribonucleic acid
N/A	not available
N/D	not determined
oriC	origin of replication
PEG	polyethylene glycol
PFGE	pulsed field gel electrophoresis
p.s.i.	pound per square inch
PTA	phosphotungstic acid
PTS	phosphoenolpyruvate:carbohydrate transfer system
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
rps	revolutions per second
SR	sensory rhodopsin
T	thymidine
Tris	tris(hydroxymethyl)aminomethane
TEM	transmission electron microscope
tRNA	transfer ribonucleic acid

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INTRODUCTION

Archaebacteria

Archaebacteria:

Until less than two decades ago, living organisms were classified into two major groups of eucaryotes and procaryotes (Stainer and van Niel, 1941, and van Niel, 1946) based on whether or not they possessed an internal bilayer membrane that contained their genetic material, the nucleus. There were many other controversial "strange" individuals and groups of living organisms that were different from members of the two well established groups and could not easily fit in either of the two groups (Jones et al, 1987 and Last, 1988). These diverse living organisms had their distinct differences and resemblances with the members of the other two groups and also had their own unique features.

Based on investigations of homology of 16S ribosomal RNA (rRNA), Woese and his associates (Woese and Fox, 1977, Woese et al, 1978, Woese and Wolfe, 1985 and Woese, 1987) introduced a new concept which rearranged living organisms into three primary lineages of Archaebacteria, Eubacteria and Eucaryotes. In this new classification, the kingdom of procaryotes was actually divided into two phylogenetically distinct groups of archaebacteria and eubacteria (Fox et al, 1980). Since then, the concept of archaebacteria as a distinct phylogenetic group has been confirmed by many genetic, molecular, physiological and evolutionary lines of evidence.

Archaebacteria have a number of common characteristics and features that make them quite distinct from eubacteria and eucaryotes such as unique DNA dependent RNA polymerase (Dennis, 1986 and Jones et al, 1987) and cell membrane (Langworthy, 1977, Kates,1978, and Kates, 1988). These groups of bacteria are distinctively different from eubacteria in terms of cytological, physiological, metabolic molecular properties and niches (Woese et al, 1978). Archaebacteria, however, display a wide range of variation among themselves (Jones et al, 1987). Based on this variation, the kingdom Archaebacteria has been divided into three groups of methanogenic bacteria, extremely halophilic bacteria, and thermophilic archaebacteria (Woese, 1987). Later based on rRNA sequence comparative studies, this classification was refined (Yang et al, 1985 and Woese et al, 1990) and the "domain" of archaebacteria was rearranged into two kingdoms of euryarchaeota, including the methanogens and their diverse relatives including extremely halophilic archaebacteria and some extreme thermophiles, and the crenarchaeota comprised entirely of extremely thermophilic archaebacteria.

Although archaebacteria show some unique physiological and metabolic specializations, their numerous similarities with the other lineages are clear indications of their divergence from a common ancestor billions of years ago (Dennis, 1986). The information stored in the genomes of living cells can be used to trace the properties of the common ancestor of all life on earth, the "progenote" which is still very poorly defined. The concept of a third lineage of life is of crucial importance in addressing questions which will clarify the properties of the progenote. A large portion of the importance of archaebacteria comes

from the fact that they obviously can provide additional information concerning evolution and related questions in procaryotes, and depict a picture of procaryotic evolution far more clear and conclusive than what eubacteria could present alone. Due to the uniqueness of many features of archaebacteria, their study will also provide valuable information which may lead to a better understanding of issues such as vertical and horizontal transfer of genes and the relation between time and molecular evolution. Comparative investigations of the three distinct lineages of living organisms that have evolved independently for billions of years, may result in a better ability to trace the pathway of evolution and possibly its mechanism.

Extremely halophilic archaebacteria:

Halobacteria are one of the most distinct groups of bacteria known so far. In Bergey's Manual, halophilic archaebacteria are grouped in the family of Halobacteriaceae. These are mainly aerobic chemo-organotrophic cells (Konig, 1988) that when monopolarly or bipolarly flagellated are motile (Alam and Oesterhelt, 1984). Some strains of halobacteria, however, can grow under anaerobic conditions by nitrate reduction (Konig, 1988).

The salt requirement of extremely halophilic archaebacteria, which in some cases is close to the saturation point, is a remarkable feature. This adaptation requires and is the result of various molecular and biochemical evolutionary changes. Extremely halophilic

archaebacteria live in media containing 3-5 M salt. They grow optimally at an NaCl concentration of 2.7-4.4 M and have a very high internal salt concentration in the range of up to 5M potassium ions. Their optimum growth temperature is between 40-45 °C (Konig, 1988) under neutrophilic or alkaliphilic (pH 8.5-9.5) conditions. Halobacterial cell walls will dissociate if the concentration of NaCl drops below 1 M NaCl causing the rod-shaped cells to become spherical (spheroplasts) (Mullakhanbhai and Larsen, 1975, Jarrell and Sprott, 1984 and Cline et al, 1989).

Halobacteria have a number of unusual and often unique features. The halobacterial features that resemble those of eucaryotes more than procaryotes are particularly interesting. Among these features are halobacterial pigments that are the only known visual pigments found in procaryotes and are similar to the visual pigments of animals (Stoeckenius, 1985). A photophosphorylation system (Konig, 1988) conducted by bacteriorhodopsin, one of the halobacterial pigmented proteins, probably represents the simplest biological method of energy coupling, synthesis of ATP and active transport (Oesterhelt and Tittor, 1989).

Why choose *Haloferax volcanii?*

Haloferax volcanii was first described as a *Halobacterium* species with moderate salt requirements isolated from the bottom sediment of the Dead Sea (Mullakhanbhai and

Larsen, 1975). *Halobacterium volcanii* was later renamed *Haloferax volcanii*, based on numerical taxonomy and polar lipid composition (Torreblanca et al, 1986). It has an optimum NaCl requirement of 1.7 M at 37 °C which increases with an increase of temperature and reaches 2.5M at 42 °C, although it can barely grow in medium containing 5M NaCl (Kauri et al, 1990, Mullakhanbhai and Larsen, 1975 and Mevarech and Werczberger, 1985). *Hf. volcanii* colonies appear pinkish-red in color. If the concentration of salt is decreased, the cells become spheroplasts and ultimately lyse. Production of spheroplasts by lowering the salt concentration has provided the basis of a PEG-mediated transformation technique used to transform *Hf. volcanii* with exogenous DNA (Cline et al, 1989). *Hf. volcanii* cells are extremely pleomorphic (Mullakhanbhai and Larsen, 1975). When grown in liquid culture under normal conditions, the cells appear circular, square, oval and triangular, commonly disc-shaped with sizes of 1-3 μm x 2-3 μm and 0.4-0.5 μm in thickness (Mullakhanbhai and Larsen, 1975).

Much of the significance of working with *Hf. volcanii* comes from the fact that compared to *Halobacterium halobium*, the most extensively studied halobacterium, it grows faster, needs a simpler defined medium (Kauri et al, 1990), is easy to maintain for daily manipulation and despite carrying many insertion elements, it is relatively more stable (Lam et al, 1990b). These and other attractive characteristics of *Hf. volcanii* such as a natural mating system (Mevarech and Werczberger, 1985), have increasingly interested researchers. This can be realized from the growing number of publications. Industrial applications of halophilic bacteria in general, such as biosensors, enzymes, and

ultrafiltration, is adding to this fast expanding area of bacteriology (Konig, 1988 and Kandler, 1984).

When this project was designed, the physical map of *Hf. volcanii* was about to be published and many details of its genes and genome organization were already being worked on. The taxis and motility mechanism was chosen as a gene pool including dozens of important genes. Cloning, mapping and sequencing these genes could add much detailed information to the *Hf. volcanii* genetic map. This information, on the one hand, would expand our understanding of the gene and genomic organization of halophilic bacteria, particularly *Hf. volcanii*, and on the other hand could be used in comparative and evolutionary studies.

Genome mapping

Physical genome mapping:

Remarkable advances have been made possible in elucidating genome and gene structures especially from bacterial cells in the past several years through invention and improvement of molecular biology and molecular genetic techniques. These techniques have made manipulation of cells and the genetic material a child's play. Thoughtful and smart approaches have increased the efficiency of researchers more than ever before in

producing detailed physical maps of bacterial genomes.

Presently, two basic approaches are being employed to construct a physical map: "bottom-up" and "top-down". In the bottom-up approach, the genome is digested and the fragments are cloned in an ordered, overlapping minimal set. In the latter approach, the mapping is done the same way a plasmid is mapped, by using PFGE of the genomic DNA digested with restriction enzymes cutting infrequently.

In the field of archaebacteriology, so far, a genome map of the halophile *Hf. volcanii* DS2 has been published (Charlebois et al, 1991), and the map of another halobacterium, *Halobacterium* sp. GRB, is near completion (R.L. Charlebois, pers. commu.). Bottom-up methods (Charlebois et al, 1991) have been used to construct these two maps. The map of *Haloferax mediterranei*, another halophile, has also been constructed, using a top-down method (López-García et al, 1992). Now, construction of the map of an average-sized bacterial genome in one year is not a dream. Physical maps provide information about the organization of the genome and also the organization of genes and operons. Detailed comparisons of maps at high resolution will help put together a universal model for explaining the mechanism that governs genome evolution.

The resolution of these maps increases as more genes are cloned and characterized. Comparison of detailed genetic data from various bacteria will reveal more and more about the relationship of these organisms and the evolution of their genes and genomes.

This information could ultimately lead to the construction of a real evolutionary tree of life with the real position of every organism.

Halobacterial genome size and structure:

Renaturation ($Cot_{0.5}$) analysis and pulsed field gel electrophoresis are the two major approaches employed to determine genome sizes in archaebacteria. These and other complementary investigations have demonstrated that archaebacterial genome size varies between 0.8 x 10^9 Dalton in *Thermoplasma* (Searcy and Doyle, 1975) to 2.4 x 10^9 Dalton in halobacteria (Moore and McCarthy, 1969). The G+C content of archaebacterial DNA ranges from 25 to 68 mol% (Schalkwyk, 1990). Halobacterial genomes possess general features that are found in eubacteria, consisting of a circular DNA chromosome and other extrachromosomal DNA materials. Extrachromosomal DNA is very common in halobacteria and comprises 11-36% of the total genomic DNA (Brown et al, 1989). The halobacterial genome also contains a variety of phages (Zillig et al, 1986).

Genomic DNA of halobacteria separates into two bands in a CsCl gradient on the basis of its mol% G+C content (Joshi et al, 1963 and Moore and McCarthy, 1969). Sixty to ninety percent of the genomic DNA is collected in a G+C-rich band with G+C content of 66-68% ("FI" DNA), and 10 to 30% of the total genomic DNA is collected in a minor band ("FII" DNA) with G+C content of about 58% (Pfeifer et al, 1982).

Plasmid variation and a high frequency of genomic DNA rearrangement which make the halobacterial genome very unstable has been noticed in halobacteria (Sapienza et al, 1982). This instability, due to insertion elements, may be the main source of halobacterial genomic DNA diversity (Pfeifer et al, 1981). Several IS elements have been identified in *Hb. halobium* ranging in size from 520 bp (DasSarma et al, 1983) to 1895 bp (Brown et al, 1989). These IS are highly mobile and may be repeated many times in the *Hb. halobium* and in the *Hf. volcanii* genome (Sapienza et al, 1982 and Sapienza and Doolittle, 1982a and b). IS elements are found in both FI and FII DNA but occur more often in A+T-rich sequences (Pfeifer, 1986 and Pfeifer and Betlach, 1985). The real role of these IS elements is not clearly understood yet.

The *Hf. volcanii* map:

The genome of *Hf. volcanii* DS2 was the first halobacterial genome to be mapped by deploying the efficient and simple "Landmark Strategy" (Charlebois et al, 1989). This produces a valuable minimal set of overlapping clones (Charlebois et al, 1991). The minimal set of the *Hf. volcanii* genome contains 151 overlapping cosmid clones covering 96% of its 4.1 Mbp genome. The *Hf. volcanii* genome consists of a circular chromosome of 2920 kbp and four plasmid replicons with the sizes of 690, 442, 86, and 6.4 kbp designated pHV4, pHV3, pHV1, and pHV2 respectively (Charlebois et al, 1991). The chromosome contains 97% FI DNA and two small AT-rich regions of FII DNA, "oases",

with frequent sites for infrequently cutting restriction enzymes. There is FII DNA in the other replicons except pHV3. The average G+C content of *Hf. volcanii* genomic DNA is 64.9 mol% (Ross and Grant, 1985, and Gutiérrez et al, 1989, and 1990).

Construction of the detailed physical map paved the way for further detailed genetic investigations of the *Hf. volcanii* genome. Efforts are underway to place many genes on the *Hf. volcanii* physical map. This is basically done by bulk probes such as tRNA's or insertion element families (Cohen et al, 1992), heterologous or homologous hybridization of cloned genes (Charlebois et al, 1991), or complementation of mutants with cosmid clone DNA (Cohen et al, 1992) or with a shot-gun library.

The genetic manipulation of halobacteria needed new tools and methods. The 6354-bp plasmid pHV2, the complete sequence of which has been determined (Charlebois et al, 1987) was used to construct *Hf. volcanii-E. coli* shuttle vectors (Lam and Doolittle, 1989 and L.C. Schalkwyk, pers. commu.). These plasmids have unique cloning sites and carry resistance to ampicillin and to mevinolin, an inhibitor of HMG CoA reductase (Cabrera et al, 1986, and Lam et al, 1990a), or carry resistance to ampicillin and trimethoprim (Schalkwyk, pers. commu.) which inhibits dihydrofolate reductase (Zusman et al, 1989). *Hf. volcanii* WFD11, a pHV2-free strain (Charlebois et al, 1987) is widely used as the host bacterium for complementation studies. These new tools and methods such as PEG-mediated transformation (Cline et al, 1989) paved the way for cloning and locating genes on the physical map.

So far, thirty cloned protein or structural RNA genes (Charlebois et al, 1991), 46 tRNA genes plus 35 amino acid and purine biosynthetic loci (Cohen et al, 1992), seven heat shock candidates and other highly expressed loci (Trieselmann and Charlebois, 1992), and 49 insertion elements (Cohen et al, 1992 and Schalkwyk et al, 1993) have been located on the *Hf. volcanii* physical map. Cloning more genes will provide further details to the map and additional useful information.

It seems that the pattern of gene location is not merely incidental, such that gene distribution has a functional pattern. This pattern is consistent with the mosaic structure of the genome consisting of patches of compositionally distinct DNA (Charlebois et al, 1991). Protein-encoding genes and tRNA genes are basically located on the chromosome whereas the insertion elements are generally identified on the plasmids. Although the plasmids make up about 30% of the genome, protein-encoding and other important genes have not yet been found on them.

Detailed comparative investigations of properties of halobacterial genomes will probably reveal very exciting information about the origin and dispersion of the different families of insertion elements that are generally found in the lower G+C content F II DNA (Pfeifer, 1986). Functions and usefulness of these highly mobile elements which are the main cause of halobacterial genome dynamism are not clear. Investigation of these insertion elements may provide useful information about their relationship and about halobacterial evolution.

With recent progress in method and tool development, resulting in rapid production of information about the genetics of halobacteria, and armed with a database to store and to analyze this information, it seems that the stage is just set for a breakthrough in molecular archaebacteriology. Mapping and comparing more halobacterial genomes will address important questions in genome and gene structure, genome and gene evolution and gene organization. At a greater scale, comparison of physical maps of archaebacteria and eubacteria will probably provide information about many basic mechanisms that control genome and gene organization. Comparative studies of the maps of procaryotes and eucaryotes may address the enigma of relative closeness of archaebacteria to eucaryotes. These comparisons will reveal the probability of existence of common features of the three lineages of life in the ancient organism that served as their ancestor.

My strategy to clone motility and taxis genes:

There are three major approaches in cloning halobacterial genes, i. complementation of mutants; ii. probing genes from recombinant clone libraries; and iii. finding genes using appropriate antibodies.

The first step in finding genes involved in taxis and motility is to isolate and characterize *Hf. volcanii* mutants defective in motility. These mutants can then be genetically complemented by a shotgun library of *Hf. volcanii* genomic DNA to clone the responsible

genes. The cloned genes would then be sequenced and used in comparative studies, which will provide additional information about archaebacterial genetics. On the other hand, studies of systems and mechanisms such as energy conversion, motility, color or chemical discrimination, sensing and signal transduction may be useful in better understanding the highly complex and almost untouchable similar systems and mechanisms in higher organisms. In this thesis, I describe progress along these lines.

Bacterial taxis and motility

Taxis and motility in Eubacteria:

The environment that accommodates bacterial populations is in steady and constant flux. Thus, it will be of vital importance for any specific bacterium to constantly adapt itself to these changes. The process of adaptation basically involves one or more systems that enable(s) a bacterium to recognize different conditions, and motility machinery that is used to approach a favourable condition and/or move away from an unfavourable one. Taxis and motility are privileges in many situations where a bacterium needs to recognize environmental changes and adapt itself properly. It is very interesting to know how this vital machinery functions and how it has evolved.

Taxis and motility systems are composed of a combination of genetic and biochemical

subsystems. Environmental changes (stimuli) are sensed by sensory receptors located on the cell surface. This information enters into a cascade of events involving phosphorylation and dephosphorylation of regulatory and signal transducer proteins (Stock et al, 1990) which delivers the generated signals to the responsive components that interpret them and respond to them properly. Such a mechanism that consists of entering a signal into a cascade of events ending with a responsive action is called signal transduction. Chemotaxis, phototaxis and aerotaxis are good examples of signal transduction. The measures that these mechanisms adopt, enables the bacterium to adapt to the environmental changes. These responses play an important role in the bacterial population dynamics and distribution (Ford et al, 1991).

How do bacteria detect the environmental signals in the form of stimuli? How do they transfer these signals to the appropriate responding apparatus? How do these relate to the motility machinery? And how does the motility machinery function? These are the very basic questions that should be addressed.

Although motility had been noticed since the early days that observation of unicellular organisms became possible, there was no or very little progress in studying taxis and motility until the end of the first half of the 20th century. Investigations pioneered by researchers such as Julius Adler in the 1960's led to a better understanding of bacterial motility and taxis (Adler and Dahl, 1967 and Adler, 1966 and 1969). Adler and his associates isolated and characterized many motility mutants of *Escherichia coli*

(Armstrong et al, 1967) and located some of the genes involved in this complicated phenomenon. Adler (1969) showed that there are specialized cellular receptors that sense specific chemicals. He suspected that phosphorylation was involved in the process of detection of stimuli and that the chemoreceptors were located on the outside of the cells. In the mid 1960s, Julius Adler and his co-workers placed the corner stones of these investigations by isolating and characterizing chemotactic mutants of *E. coli* (Adler,1966, Adler and Dahl, 1967, Adler and Templeton, 1967, Armstrong et al, 1967, Armstrong and Adler, 1969a and 1969b, Parkinson, 1976). They reported that cell surface receptors detect chemical changes in the environment and pass them to the motility machinery by low molecular-weight molecules that function as signal transmitters involving phosphorylation reactions. They also documented that the proton motive force is utilized to drive the cell back and forth by a CW or CCW rotation of the flagellar bundle (Adler, 1975). Yet, the biochemical and molecular investigations of chemotaxis and motility had to await appropriate tools to be developed.

The most detailed taxis and motility information comes from investigations carried out on chemotaxis in *E. coli*, the best genetically known microorganism. *E. coli* has 4-8 flagellar filaments grown randomly on its body (peritrichous flagellation). These flagellar filaments form a left-handed semi-rigid helical structure due to mechanical and hydrodynamic forces. Rotation of this bundle pushes the bacterium forward. When the sense of rotation changes, the flagellar filaments fly apart and the bacterium tumbles (Block et al, 1991, Macnab, 1987 and Parkinson and Hazelbauer, 1983). In a uniform

environment, *E. coli* performs periods of random tumbling and straight swimming in all dimensions (Macnab, 1987). Once a stimulus is introduced, there is no response in the first 0.2 seconds and then the frequency of tumbling and flagellar rotation deviate from their normal values (Segall et al, 1982). In this state of excitation, CW and CCW rotation of the flagellar motor contributes to the net progress towards a favourable stimulus or escape from an unfavourable one. The new values of tumbling and flagellar motor rotation and the length of the excitation state depends on the nature and strength of the stimulus and the extent of its binding to the membrane receptors (Parkinson and Hazelbauer, 1983 and Macnab, 1987). During the adaptive state which follows the excitation state, the tumbling and flagellar motor rotation frequencies gradually return to their pre-stimulus values and the cell returns to the normal pattern of swimming. It seems that *E. coli* is always comparing the present situation with the former one (Segall et al, 1986) and adopts appropriate measures in adapting to the new conditions.

E. coli cell senses the presence of a newly introduced stimulus when it binds to its transmembrane receptors (Parkinson and Hazelbauer, 1983). The interaction between membrane receptors and their ligands produces a signal (Macnab, 1987). It has been proposed (Asakura and Honda, 1984) that the transmembrane receptors exist in two equilibrated forms of S and T. Binding of a repellent to the S form causes its methylation by a transferase and binding of an attractant to the T form receptor causes its demethylation (Segall et al, 1986). A family of methyl-accepting proteins seem to be involved in the S and T form equilibrium. Several of these membrane receptors that are

about 60000 Mr molecules, and undergo methylation and demethylation, have been identified (Springer et al, 1977, Silverman and Simon, 1977, and Kondoh et al, 1979). The signal produced by the transmembrane receptors is relayed to their cytoplasmic counterparts which function as molecular messengers. These messengers are transducer proteins that carry the signal from the membrane receptors to the appropriate responding components by transferring phosphoryl groups from histidine to aspartate residues (Stock et al, 1990). CheY is one of the best known transducers whose activity is controlled by membrane receptors and is responsible for CW rotation of the flagellar motor (Macnab, 1987, and Barak and Eisenbach, 1992b). The role of other signal transducer proteins, such as CheA and CheZ, is not clear yet. Like transmembrane receptors, signal transducer messenger molecules may also be signal-specific, each responsible for carrying a specific signal. Size similarities, conserved residues and high frequency of homology in the N-terminal has been noticed among protein transducers that have so far been identified and characterized in different bacteria (Bischoff and Ordal, 1992). This may mean that these bacteria have received these similar machineries from their common ancestor before deviating from it. The existing differences and unique features may be the result of long term individual evolutionary adaptations.

The flagellum and its components:

The intact flagellum consists of an external part, the flagellar filament, which is about 5-

10 μm long and 20 nm in diameter (O'Brien and Bennett, 1972). The flagellar filament consists of thousands of identical protein monomers with Mr of 55,000 (Kondoh and Hotani, 1974) and is connected to the cell through a curved structure called the hook (Aizawa et al, 1985). The hook functions as a flexible joint and is structurally similar to the external part of the filament but consists of protein monomers of Mr 42,000 (Wagenknecht et al, 1982).

The flagellar filament and the hook portion are connected to the cell envelope by a complex structure consisting of a set of four rings, through which a shaft-like rod passes (Schuster and Baeuerlein, 1992). This structure is called the basal body which is about 25 nm in diameter, anchored to the cell envelope by a larger (70-200 nm) disk (Schuster and Baeuerlein, 1992). Two of the rings, L and P, form the outer part of the basal body. The L ring is located in the outer membrane and the P ring is located in the peptidoglycan layer (Schuster and Baeuerlein, 1992). These rings are surrounded by force-generating molecules (Miester et al, 1989). The other two rings, called S and M, serve as a mounting base. At least seven proteins are associated with the basal body (Macnab, 1987). Among these are MotA and MotB torque generating proteins, which have been identified near the M ring (Stolz and Berg, 1991) and have been suggested to function as proton channel and linker of the cell wall respectively (Stolz and Berg, 1991). The torque generated by Mot proteins is transferred to the external filamentous portion of the flagellum through the shaft.

Flagellar motor and switching:

Each flagellar filament is propelled by an individual rotary motor located at its base (Meister et al, 1987). These motors do not need ATP and use membrane potential energy to function. Signals, transmitted from transmembrane receptors, control the sense and rotation frequency of each motor (Meister et al, 1989) by interacting with the switch factor. The nature of switching and its factor(s) are not yet known very well. It has, however, been documented (Barak and Eisenbach, 1992a) that fumarate restores switching ability in *E. coli* and *Salmonella typhimurium* mutants defective in switching. Three proteins, FliG, FliM, and FliN have been identified to be involved in the switching in *E. coli* and *S. typhimurium* (Sockett et al, 1992 and Francis et al, 1992). These proteins form a structure that is involved in energizing and switching of the flagellar motor (Khan et al, 1992). The FliG protein has been identified as one of the switch factors attached to the M ring (Francis et al, 1992).

Genetics of motility:

With the advancement of molecular genetic techniques, especially in the past decade, our knowledge of bacterial genetics has increased very rapidly. A variety of approaches have been employed to investigate the genetics of bacteria. Mutants have been an important tool in these studies and have played a crucial role in revealing the facts about genetics

of bacteria. About 60 genes have been reported (Macnab, 1987) to be involved in motility and chemotaxis in *E. coli* and *S. typhimurium*. At least 30 of these genes in *E. coli* (Parkinson and Hazelbauer, 1983) and 40 genes in *Caulobacter* (Ramakrishnan et al, 1991) are involved in flagellar construction, assembly and functioning. Flagellin, the protein that constitutes the flagellar filament, is encoded by a single gene (Macnab, 1987) in *E. coli* and two genes in *Caulobacter* (Gill and Agabian, 1983a and 1983b). Studies with *E. coli* have demonstrated that the flagellar assembly process takes place gradually from proximal to distal; i.e. it begins with the assembly of components located in the membrane. The outer rings, hook and the flagellar filaments are assembled next. Assembly of the M and S rings and the rod needs at least 16 genes (Macnab, 1987). The presence of divalent cations such as calcium is crucial in this process (Robinson et al, 1992). The assembly dissociates if the concentration of cations is lowered. There is not enough information about many aspects of this process such as assembly of the proteins that connect the rings, the hook and its accessory proteins and different aspects of the assembly of the flagellar filament itself. The mechanism that determines the length of flagellar filaments, which may be controlled mechanically through fractionation or genetically by products of the *flaEe / flaRs* genes, is very poorly defined (Macnab, 1987).

The genes, involved in the synthesis, assembly and functioning of flagella are generally found in two clusters at 41-43 min on the physical map of *E. coli* (Macnab, 1987). One of these clusters contains genes involved in flagellar synthesis. About 33 genes are under the control of the *flbB / flaI* main regulatory operon which are in turn under the control

of a complex of cyclic AMP/CAP (Macnab, 1987). Investigation of different motility-defective mutants may reveal more undiscovered genes and provide more information about the role of the related genes.

Taxis and motility in halobacteria:

Chemotaxis, phototaxis, or aerotaxis have been reported for many archaebacteria (Grogan, 1989, Sment and Konisky, 1989 and Bibikov and Skulachev, 1989). Most of the related investigations, however, have been carried out on phototaxis of *Hb. halobium*, the most extensively studied halobacterium. Much of the attraction of halobacteria comes from their unique, simple and very efficient system of energy production which is capable of converting light energy to mechanical and chemical energy. Photophosphorylation, oxidative phosphorylation, and substrate-level phosphorylation have been reported in halobacteria (Konig, 1988).

The mosaic membrane of halobacteria plays a very important role in responsive reactions and energy generation. Different pigment-containing acidic proteins that function as membrane receptors and which control aerotaxis, phototaxis and chemotaxis are responsible for this mosaic structure (Sumper and Herrmann, 1978, Alam and Oesterhelt, 1984). Four major families of these pigmented proteins have so far been identified in *Hb. halobium*: bacteriorhodopsin (BR), a light-driven membrane proton pump (Oesterhelt and

Stoeckenius, 1971 and Stoeckenius, 1985) which functions as a light transducer photoreceptor with Mr of 40,000 (Blanck et al, 1989), halorhodopsin (HR) (Traulich et al, 1983), a light-driven chloride pump with Mr of 25000 (Oesterhelt and Tittor, 1989, Blanck et al, 1989), and two sensory rhodopsins (SR-I and SR-II (Bogomolni and Spudich, 1982, Spudich and Bogomolni, 1984 and Otomo et al, 1989)), which are involved in phototaxis and color discrimination (Bibikov and Skulachev, 1989, Bogomolni and Spudich, 1987 and Marwan and Oesterhelt, 1987). These molecules are composed of a protein unit which forms a Schiff's base by covalently binding to a retinal pigment through a lysyl residue (Bogomolni and Spudich, 1987). Recently another retinal protein, designated archaerhodopsin, has been reported from *Halobacterium* sp. aus-2 (Uegaki et al, 1991) which functions as an outward proton pump. It should not be a big surprise if more pigmented proteins are identified in diverse members of archaebacteria.

BR, which consists of seven alpha helices and makes up 75% of the halobacterial purple membrane (Sumper and Herrmann, 1978), is activated by small fluctuations in the proton motive force (Baryshav et al, 1981). The energy generated by the activation of BR is used in the synthesis of ATP, in active transport and in flagellar rotation (Bogomolni and Spudich, 1982). Converting light energy to mechanical energy is an interesting feature of halobacteria.

Slow Cycling Rhodopsins (SR's) (Bogomolni and Spudich, 1982), with a photoreaction cycle hundreds of times slower than those of BR and HR are the bases of colour

discrimination in halobacteria. These pigments sense attractant and repellent lights and thereby enable the cell to control its swimming pattern to find suitable light (green) and to avoid harmful light (blue). SR-I functions as a long-wave (red) light attractant at 573 nm and a short-wave light repellent at 373 nm with a photoreaction cycle half-life of one second (Bogomolni and Spudich, 1982). SR-II, also called phoborhodopsin or P480 (Marwan and Oesterhelt, 1987), functions as a short-wave (blue and near UV) light repellent (Spudich and Bogomolni, 1984 and Bogomolni and Spudich, 1987). SR-II is present at all stages of growth. SR-I, however, seems to be inducibly constructed and appears in the mid-log phase (Otomo et al, 1989).

Attractant and repellent signals, generated by activation of pigmented molecules by light (Marwan et al, 1988), are picked up by specific methyl-accepting transmembrane receptor proteins and transmitted through specific pathways (Alam et al, 1989 and Spudich et al, 1988). Methylation of specific glutamyl residues activates these proteins (Alam and Hazelbauer, 1991). Different families of methyl-accepting proteins are involved in transmitting signals to the responsive organelles. Seven families of these transmitters ranging from 65 to 150 KDa (Spudich et al, 1988 and Alam and Hazelbauer, 1991) have been identified. A group of methyl-accepting proteins have been proposed to be the trigger of SR-I activation which converts photon absorbtion by the pigment to protein conformational changes (Spudich et al, 1989 and Yan et al, 1991b). A 94 KDa protein resembling a chemotaxis signal-generating protein in *E. coli* and another 25 KDa protein have been found to be associated with SR-I (Spudich et al, 1989). The specific role of

these proteins are not clear yet. An increase in an attractant signal suppresses the flagellar reversal and an increase in a repellent signal elevates the flagellar reversal (Spudich and Bogomolni, 1984). It seems that all of the transmitted signals fall into a unique pathway before reaching the flagellar switch factor which has been identified as fumarate in *Hb. halobium* (Marwan et al, 1991).

Flagella in halobacteria:

Investigation of flagella and their components in halobacteria is very interesting since they form an important part in a complex machinery that converts light energy to chemical and mechanical energy. *Hb. halobium* cells are basically mono-polarly flagellated but in the stationary phase, the cells become bipolarly (lophotrichously) flagellated (Alam and Oesterhelt, 1984). Flagellar filaments in *Hb. halobium* are 4 μm long (Houwink, 1956) and contrary to eubacteria (*E. coli*), form a right-handed helical structure (Alam and Oesterhelt, 1984). Each bundle consists of 5-10 filaments (Houwink, 1956) and is able to rotate in both directions (Schimz and Hildebrand, 1985). The flagellar filaments do not fly apart when the sense of rotation changes. In bi-polarly flagellated cells, the flagellar bundle is longer on one pole than on the other pole (Alam and Oesterhelt, 1984). A large amount of about 30 μm long loose flagellar filaments have also been noticed in *Hb. halobium* (Dundas, 1977) the significance of which is not clear yet.

Hb. halobium swims bidirectionally and tumbling is absent between alternations of the flagellar rotation sense (Alam and Oesterhelt, 1984). Mono-polarly flagellated *Hb. halobium* cells swim forward, stop and then continue forward or reverse their direction of swimming (Alam and Oesterhelt, 1984). CW rotation of the flagellar bundle drives *Hb. halobium* forward and CCW rotation of the flagellar bundle generates a force that pulls the cell backward. The swimming pattern in bi-polarly flagellated cells is similar to that of mono-polarly flagellated cells if one of the bundles stops. Opposite rotation of the two flagellar bundles drives the cell from right to left or from left to right. Operation of the two flagellar bundles is not coordinated (Oesterhelt and Marwan, 1987). The sense of flagellar rotation changes spontaneously every 10-20 sec (Schimz and Hildebrand, 1992). The frequency of change of the rotation sense deviates from normal values upon introduction of a stimulus. Increase in blue light and/or decrease in red light (unfavourable) increases this frequency. In contrast, an increase in red light and/or a decrease in blue light (favourable) decreases this frequency. There is a latency phase of 0.70±0.14 sec before the normal rotation pattern alters (Sundberg et al, 1986). This time may be needed for the formation and transmission of a signal to the flagellar motor (Marwan and Oesterhelt, 1987). Once the receptors have sensed the illumination, a membrane potential is generated by BR pumping protons out enhancing the proton motive force and the thermodynamic potential which is used to synthesis ATP and energize active transport (Oesterhelt and Tittor, 1989). Following this, a state of excitation occurs during which flagellar reversal frequency increases. The excitation period lasts for tens of seconds and after that the cell becomes adapted to the new situation and the reversal

frequency returns to its pre-stimulus values. An endogenous oscillator controlling flagellar reversal has been proposed to exist in *Hb. halobium* (Schimz and Hildebrand, 1992). Incoordination of flagellar rotation in bi-polarly flagellated cells indicates that the produced signal decays very fast before reaching the distal flagellar motor. There is a pausing state between every change of rotation sense during which the speed of rotation decreases sharply (Marwan et al, 1991). It takes 2 sec before the normal speed of 2 μm per sec (Macnab and Ornston, 1977, Houwink, 1956 and Alam and Oesterhelt, 1984) is achieved (McCain et al, 1987). The size and the strength of the stimulus do not affect this period (Marwan et al,1991). Thus, it seems that an endogenous mechanism is involved in restoring normal speed after each pausing state (McCain et al, 1987).

Hb. halobium flagellar filaments are made of three different sulphated glycoproteins (Wieland et al, 1985). The Mr of these proteins, designated FlaI, FlaII, and FlaIII are 23,500, 26,500, and 31,500 respectively (Alam and Oesterhelt, 1984 and Alam and Oesterhelt, 1987). Five flagellar genes have been probed in *Hb. halobium* by antibodies, produced by using flagellar proteins as antigens (Alam and Oesterhelt, 1984). Two of these genes, that have been sequenced (Gerl and Sumper, 1988), are found in one locus and the other three are situated at another locus. Flagellar genes show a high degree of homology. The gene encoding SR-I, one of the membrane pigmented proteins, has also been identified (Blanck et al, 1989).

Similarities found among taxis components in eubacteria and to some extent between

halobacteria and eubacteria (such as fumarate as switching factor and the 94 KDa protein in *Hb. halobium*) suggest homology. Cloning more halobacterial genes and comparing them with their eubacterial counterparts will provide new information about their common ancestor and also, more broadly, about the evolution of genes and genomes in the members of these sister kingdoms.

Materials and Methods

Standard and general techniques:

I have used Sambrook et al (1989) for most of the general procedures such as plasmid DNA preparation, digestion, ligation, agarose gel electrophoresis, Southern transfer, hybridization, transformation, electroporation and screening. Wherever applicable, I will explain if I have used a different or modified method or technique.

Strains and growth conditions:

Hf. volcanii strain DS2 (Mullakhanbhai and Larsen, 1975) and WFD11 (Charlebois et al, 1987) were obtained from W.F. Doolittle, Dalhousie University, Halifax, Nova Scotia.

The cultures were grown in a medium containing 125 g NaCl, 45 g $MgCl_2.6H_2O$, 10 g $MgSO_4.7H_2O$, 10 g KCl, and 1.34 g $CaCl_2.2H_2O$ (Low Salt Medium) and 5 g Bacto tryptone (Difco Laboratories) and 3 g of Bacto yeast extract (Difco) per litre. The food and salt solutions were autoclaved (20 min at 15 p.s.i.) separately and then were mixed. The regular plates had an additional 2% and swarm plates had an additional 0.4% Bacto agar (Difco) respectively. The liquid cultures were incubated at 37 or 42 °C with a shaking speed of 100 rpm. The plates were incubated at 37 °C.

After transformation of *Hf. volcanii* cells (Cline et al, 1989), the spheroplasts were grown in low salt swarm plates containing an additional 15% sucrose for regeneration. Other growth conditions of *Hf. volcanii* were as described (Cline and Doolittle, 1992). The low salt medium plates contained 30 µM of Mev (a kind gift of Merck Sharp & Dohme Research Lab, Merck & Co., Inc., USA) or 6 mg/L trimethoprim (Sigma) for selection of transformed cells.

Maintenance and growth conditions for *E. coli* strains JM110 and DH5α were as described (Messing, 1983). The medium contained 8 g Bacto tryptone (Difco), 5 g Bacto yeast extract (Difco) and 5 g NaCl per litre. In addition, the plates contained 15 g Bacto agar (Difco) per litre. YT- ampicillin plates contained 70 mg of ampicillin (Boehringer Mannheim GmbH) per litre.

Isolation of *Hf. volcanii* motility mutants:

To isolate motility-defective mutants, first, a diluted *Hf. volcanii* culture grown to log phase was poured with 0.4% agar medium (swarm plates). A motile colony was picked and grown to log phase. In this way, I made sure that I had a motile population to begin with. This culture was poured with swarm medium. When each colony formed a swarm, 200 swarms were chosen randomly. Cells from the central regions of each of these colonies were transferred to a new swarm plate either by using a toothpick or by injecting

a few μL of cell suspension. When these new inocula formed swarms, cells from their central regions were transferred independently into new swarm plates by stabbing with a toothpick or by injecting with a pipette. These serial transfers of cells from old plates to new plates was done 10 to 12 times for each of the 200 colonies until the colonies grew very small compared to the wild-type colonies in the same period of time.

The twenty-five slowest-growing colonies were chosen. An independent liquid culture was prepared from each of these twenty-five samples and poured with swarm medium. When a few colonies on each plate demonstrated motility characteristics and formed swarms, the colonies that were still not motile were chosen as non-motile mutants for they had been given enough time to form swarms if they had contained any motile cells. Eight colonies from each of the twenty-five samples were picked, grown, and stored at -80 °C with 15% glycerol for further investigation.

Using the same serial transferring method, four independent mutants were isolated from the edges of the grown colonies. Swarms of these mutants that I have called super-motile grow much faster than swarms of wild-type *Hf. volcanii*.

Characterization of motility mutants:

A log-phase culture of each mutant was poured with swarm medium to study colony

appearance. Stability and rate of reversion of the mutants was estimated from the ratio of motile to non-motile colonies on the swarm plates.

Further characterization of *Hf. volcanii* strain DS2 and the motile defective mutants was carried out by electron and light microscopy.

Light Microscopy:

A drop of culture, or of cell suspension, from different stages of growth was placed on a microscope slide. The slide was covered with a glass cover and was sealed with vaseline. A Wild phase contrast microscope set at 100X magnification was used to observe the cells.

Electron microscopy:

The cells were grown in liquid medium with shaking and were fixed in 10% glutaraldehyde for several hours. Ten microliters of the fixed cell culture was placed on a 400-mesh nickel grid coated with 3% parlodion and were either stained with 2% PTA or molybdate directly or after being washed with 2 or 3 drops of water. In a different method, fixed cells were shadowed with a carbon/platinum electron gun at a 10 degree

angle. The grids were observed with a Phillips 202 transmission electron microscope at 60 kV.

Genomic DNA and plasmid DNA preparation:

Hf. volcanii total genomic DNA was extracted as described (Lam and Doolittle, 1992). Another extraction method involved resuspending cell pellets in 1 M NaCl and 50 mM $MgCl_2$ and lysing them with 50 mM Tris, 50 mM EDTA and 0.2% N-lauroyl sarcosine. Before phenol extraction and ethanol precipitation, the cell lysis was digested with 0.05 mg/mL of RNase at 37 °C for 30 min and then with 0.1 mg/mL of proteinase K for 1.5 h at 37 °C (Charlebois et al, 1987). *Hf. volcanii* and *E. coli* plasmids were prepared by alkaline extraction.

Construction and amplification of a shot-gun library:

Hf. volcanii DS2 total genomic DNA was digested with MluI (New England BioLabs) and ligated to MluI-linearized pWL101 (Lam and Doolittle, 1989) or pLS46-8 (L.C. Schalkwyk, pers. comm.), *E. coli- Hf. volcanii* shuttle vectors. Both vectors are based on pHV2, a 6.4 kbp endogenous plasmid of *Hf. volcanii* DS2, the entire sequence of which has been determined (Charlebois et al, 1987). The 15 kbp pWL101 has a unique MluI site

and carries an *E. coli* replicon with an ampicillin resistance gene, and for halobacterial selection, a mevinolin resistance gene. Mevinolin is a competitive inhibitor of the enzyme HMG CoA reductase (Lam and Doolittle, 1992) and is very effective in inhibiting *Hf. volcanii* growth. pLS46-8 which became available during the progression of this project, is a 10.13 kbp plasmid carrying a multi-cloning site with an MluI site, *E. coli* and *Hf. volcanii* replicons and a tmp resistance gene from *Haloarcula marismortui* (L.C. Schalkwyk pers. commu.).

Ligations of the *Hf. volcanii* DS2 genomic DNA and linearized shuttle vectors were used to transform *E. coli* strain JM110 which is a dam$^-$ strain to overcome an *Hf. volcanii* restriction system (Holmes et al, 1991). The transformed colonies were also used to check the size of insertions.

Transformation of *Hf. volcanii:*

Transformation of *Hf. volcanii* was done as described (Charlebois et al, 1987, and Cline et al, 1989) except for the regeneration plates. For this purpose, the potentially transformed cells were resuspended in swarm medium with 2 mg/L tmp and 15% sucrose at about 50 °C and were poured into empty petri dishes.

RESULTS

Appearance of *Hf. volcanii* swarms on agar medium:

When motile bacteria are placed in a medium containing an appropriately dilute concentration of agar, they swim outward and form a swarm (motile colony). A motile colony continues growing until inhibiting environmental factors such as nutrient limitation or the accumulation of wastes decrease swimming speed of the bacterial population and the growth of the swarm. These swarms may develop patterns that are usually characteristic of a specific bacterial population.

Hf. volcanii cells swim outward when they are placed in a medium containing 0.4-0.5 % agar (swarm medium) forming a swimming colony (swarm) (Figure 1). The swarm will continue growing until it eventually covers the petri dish. Motile colonies of *Hf. volcanii* inhibit the growth of other swarms once they reach each other (Figure 1). Production of rings within motile colonies may be due to the depletion of specific nutrients (Armstrong et al, 1967 and Budrene and Berg, 1991). Wild type *Hf. volcanii* swarms appear pink in color due to their membrane pigments.

In a medium containing 1-2% agar, *Hf. volcanii* produces non-motile colonies. Sizes of these colonies vary between one to several mm. The colonies that are inside the agar

Figure 1

***Hf. volcanii* DS2 swarms in medium containing 0.4% agar**

Cells were first grown in liquid culture, then were diluted and poured with hot agar medium. Notice the pattern of rings and the inhibition of swarms when colonies approach each other (arrows).

medium usually grow slower and smaller in the same period of time than those that grow on the surface of the agar medium.

Motility mutants and their role:

Motility mutants have been a very useful tool in finding and studying motility genes. These mutants fall basically in three category (Macnab, 1987): i. mutants with no flagella or defective flagella (Fla^-), ii. mutants with paralysed flagella (Par^-), and iii. mutants with normal flagella but defective in chemotaxis (Che^-).

Isolation of *Hf. volcanii* motility-defective mutants:

Hf. volcanii strain WFD11 (Charlebois et al, 1987) was grown to log phase and poured with swarm medium to isolate single cell colonies. Two hundred independent swarms (motile colonies) were randomly picked and transferred to new swarm plates. Cells from the centers of grown swarms were transferred to new swarm plates for 10-12 times successively. By then, swimming was considerably reduced.

Cells from centers of 25 of the smallest independent swarms were grown in separate liquid cultures and poured with hot swarm medium to isolate clonal colonies. When some

of the colonies showed swimming characteristics, the colonies that were still sharp and solid were identified as non-motile colonies. Eight separate colonies from each of the 25 independent samples were picked as motility-defective mutants and stored in 15% glycerol at - 80 °C for further investigation.

Isolation of *Hf. volcanii* super-motile mutants:

Hf. volcanii super-motile mutants were isolated by a serial transfer of cells from the edge of swarms to new plates containing swarm medium. After 10-12 transfers, four independent samples were grown to log phase and stored separately as super-motile mutants in 15% glycerol at - 80 °C. Super-motile mutant swarms grow much faster than wild-type *Hf. volcanii* swarms (Figure 2).

Colony appearance of *Hf. volcanii* motility defective mutants:

Motility defective mutants produce colonies that appear different from the wild-type swarms. This appearance is characteristic of different groups of mutants which can basically be divided into three categories: i. Mutants whose colonies, on swarm plates, appear like wild type *Hf. volcanii* colonies on regular agar plates i.e. small in diameter with solid sharp edge (Figure 3); ii. Mutants whose colonies are small in diameter, 2-5

Figure 2

***Hf. volcanii* super-motile swarms**

Swarms of an *Hf. volcanii* super-motile mutant (RF202) that are running into and inhibiting (arrows) each other. These swarms are similar to wild-type *Hf. volcanii* swarms except that they grow faster and become larger in a shorter period of time. Compare these swarms with *Hf. volcanii* wild-type swarms of the same age (Figure 1).

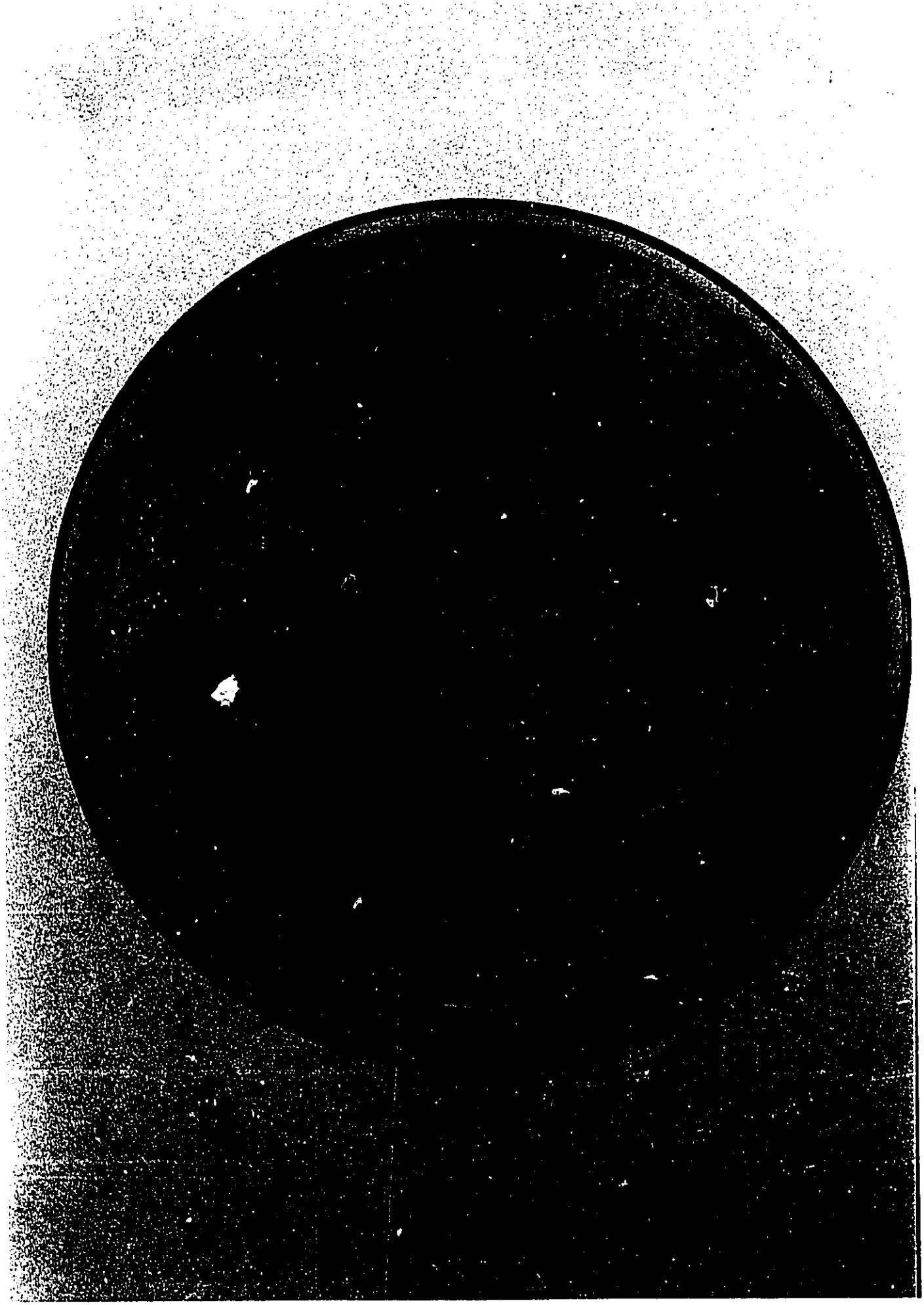
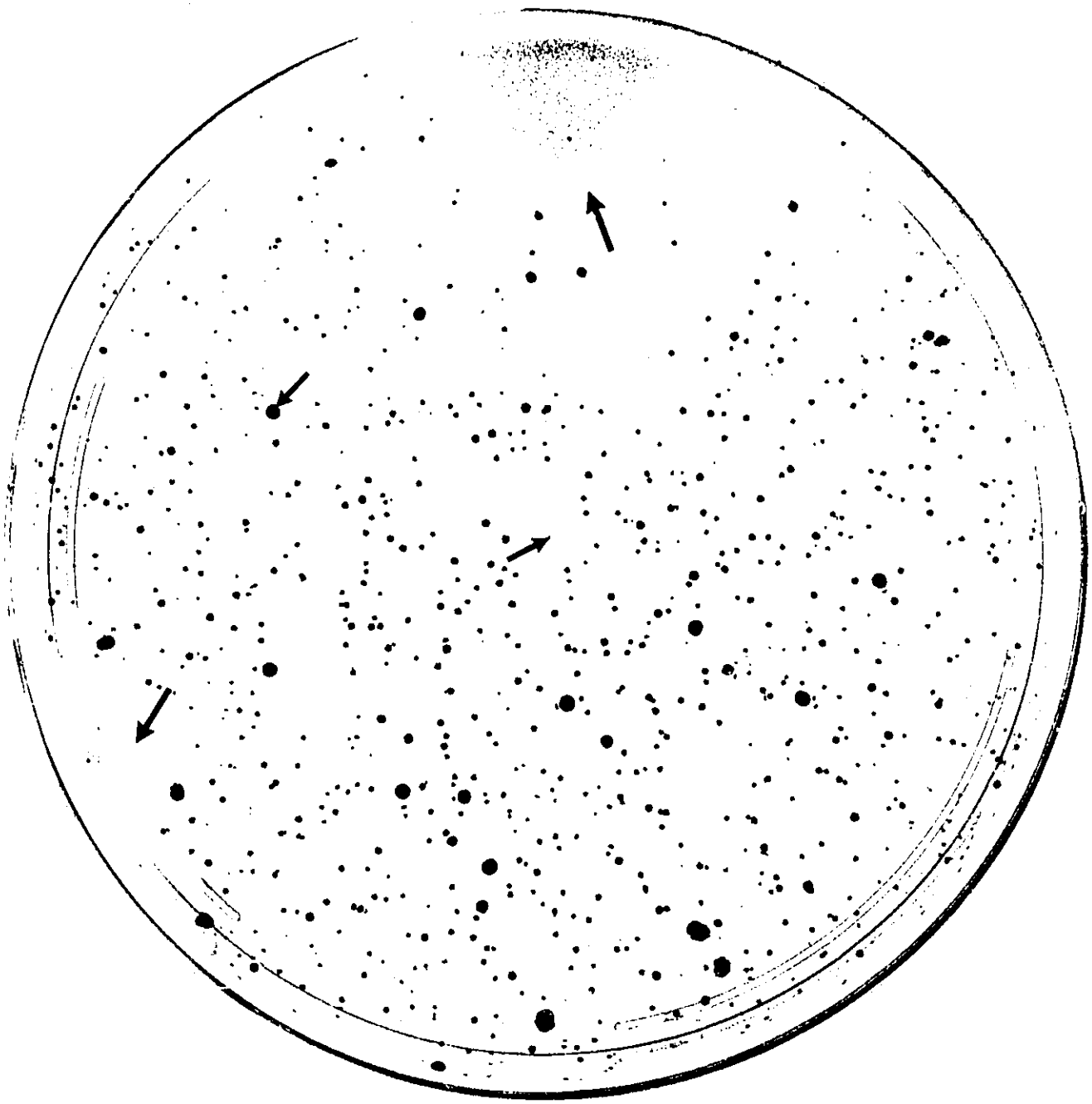


Figure 3

Colonies of the non-motile mutant (RF148) of *Hf. volcanii*

Non-motile mutants of *Hf. volcanii* form spot-like colonies (small arrows) with solid and sharp edges on swarm medium containing 0.4-0.5% agar. The picture shows a plate with non-motile colonies. The swarms that have covered a large part of the plate (large arrows) are colonies of reverted mutants.



mm, with a fuzzy appearance (Figure 4); iii. Mutants whose colonies are quite variable in size and include non-motile colonies to large swarms. Revertants of these mutants may produce swarms with a scalloped edge due to frequent and successive reversions (Figure 5). These reversions sometimes produce a dotted region in an already growing colony.

Reversion frequency:

To measure the stability of the motility defective mutants, a diluted culture of each mutant was grown to log phase and poured with hot swarm medium. The rate of reversion of each mutant was estimated from the ratio of motile to non-motile colonies. The results obtained from this test are summarized in Table 1.

Light microscopy:

Under the phase contrast microscope, wild-type *Hf. volcanii* DS2 appears as a short, motile, kidney-shaped bacterium with no obvious polymorphism through different stages of growth. It spins in both CW and CCW direction and is able to switch the sense of its rotation. The motility pattern of *Hf. volcanii* as far as one could perceive under the light microscope consists of rotating, stopping and revesing the sense of rotation. Between each

Figure 4

Colonies of the filamentous *Hf. volcanii* mutant (RF129) in swarm medium

Mutants that predominantly grow longer without dividing (as observed under the light microscope) form small swarms that grow for a while and then stop. These swarms are fuzzy and usually have no rings.

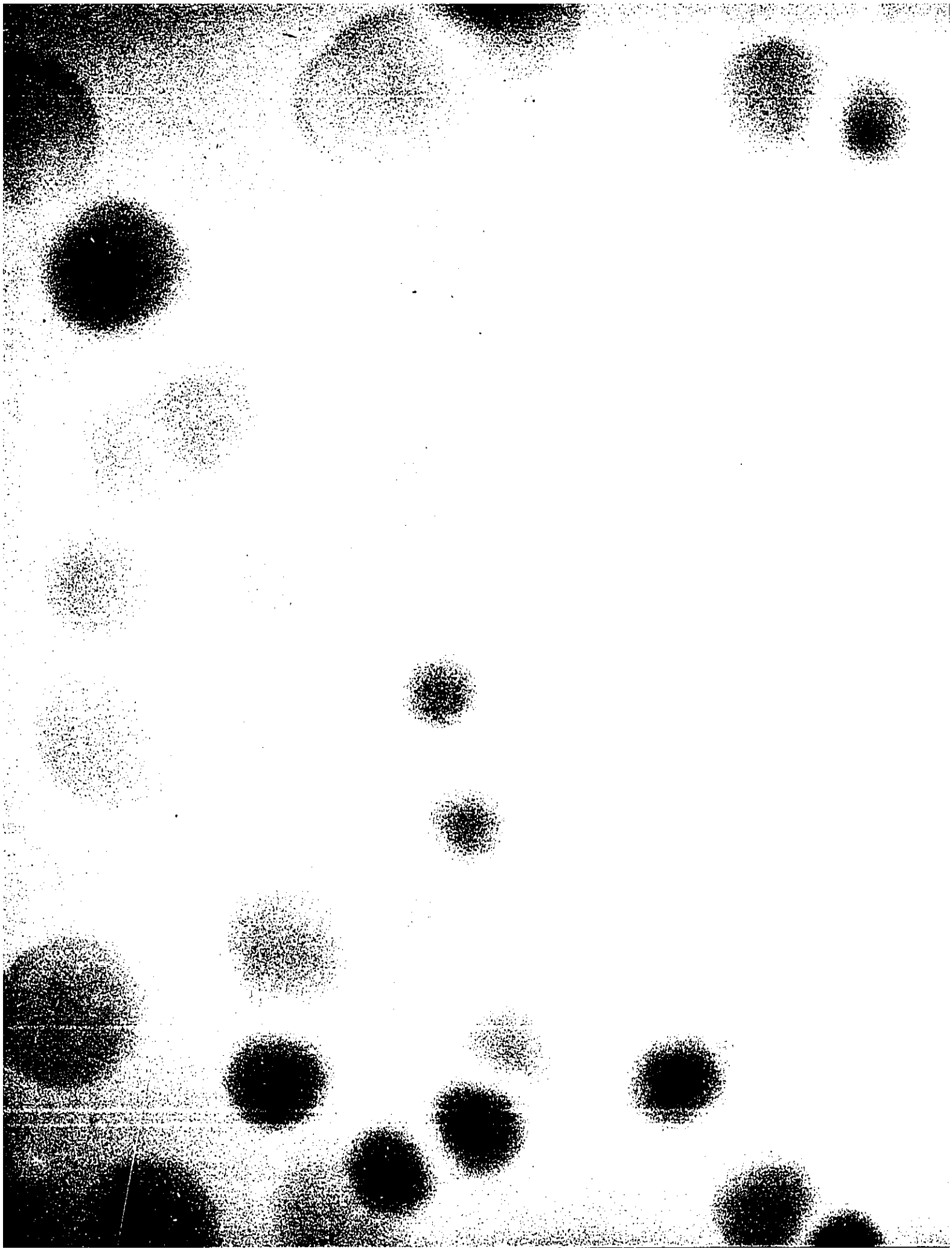
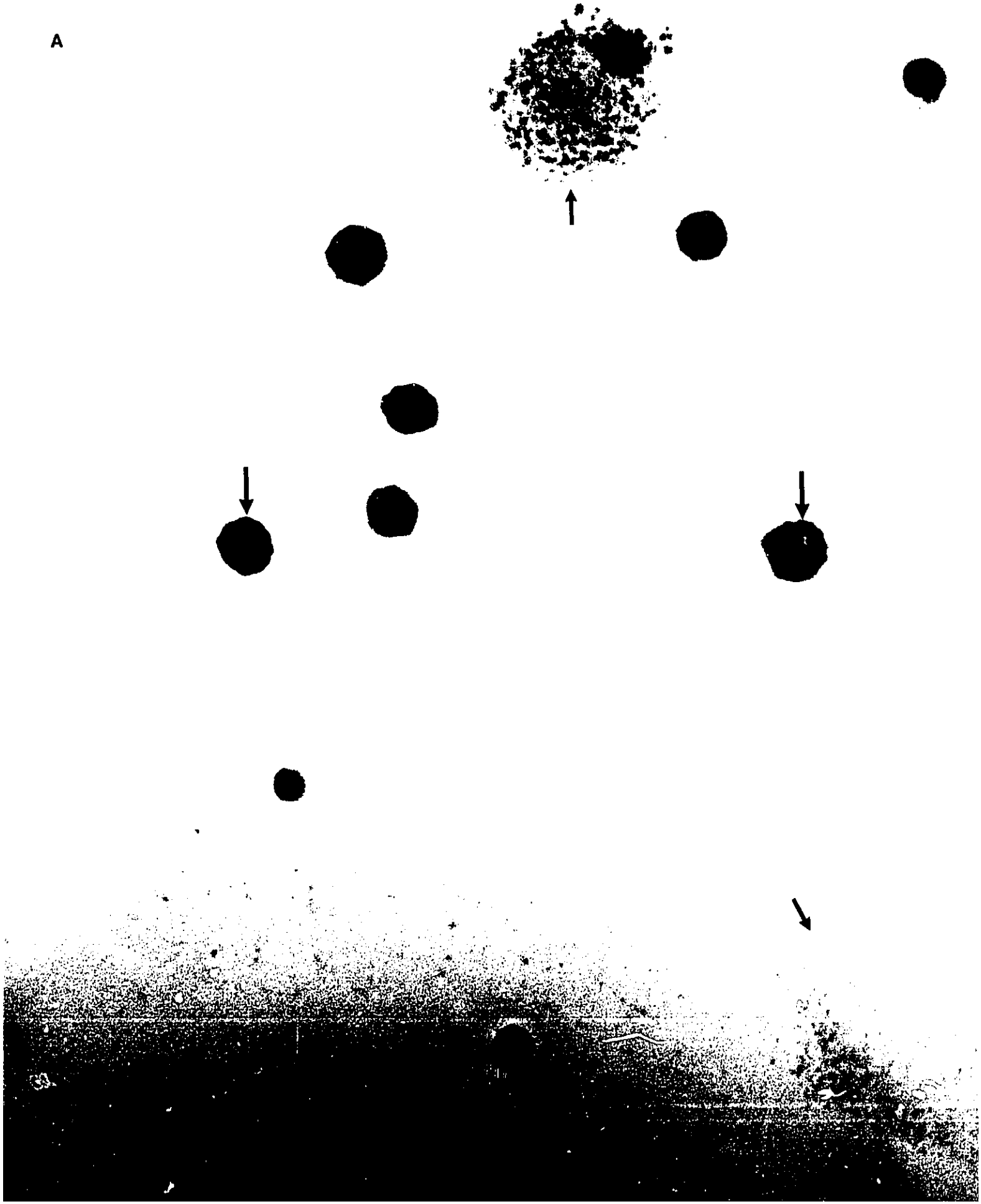


Figure 5

Dotted swarms of frequently reverting *Hf. volcanii* motility mutants

RF160 (panel A) and RF178 (panel B) non-motile mutants (large arrows) frequently produce revertants whose swarms have a dotted, scalloped edge (small arrows) in swarm medium containing 0.4-0.5% agar.


A

B

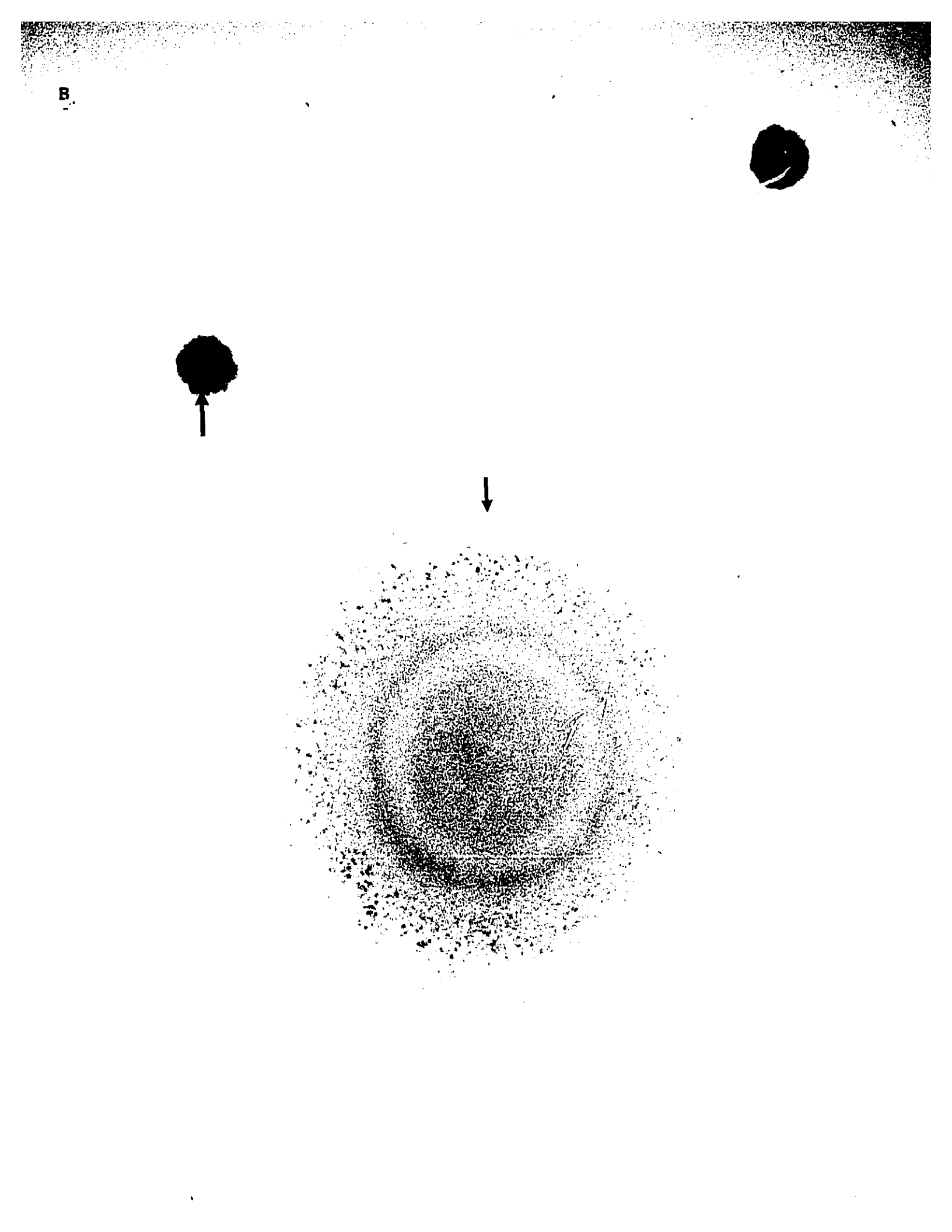


Table 1:

Reversion frequencies

In this table, I have summarized the results of reversion rates obtained from pouring a diluted culture of each of the motility defective mutants in swarm medium and calculating the ratio of motile to non-motile colonies for each of the mutants. As it is realized from these results, the motility defective mutants are basically divided into three major groups: i. the mutants that have sharp and non-motile colonies with no or a small rate of reversion; ii. the mutants with small fuzzy colonies; and finally iii. the mutants, the colonies of which are variably motile.

Legend:

NA Results were not available at the time.

* All colonies small and fuzzy.

** Mixture of sharp colonies and variable-sized swarms.

No.	Strain	Total colony #	# of swarms		% of swarms	
			day 7	day 14	day 7	day 14
1	RF115	1322	0	0	0	0
2	RF148	1152	0	0	0	0
3	RF189	1220	0	0	0	0
4	RF177	560	0	0	0	0.17
5	RF188	1100	0	2	0	0.18
6	RF178	1200	0	3	0	0.25
7	RF154	1640	0	5	0	0.30
8	RF181	320	0	1	0	0.31
9	RF113	360	0	2	0	0.54
10	RF160	365	0	3	0	0.82
11	RF169	108	NA	**	NA	**
12	RF120	112	*	*	*	*
13	RF151	122	*	*	*	*
14	RF187	106	NA	*	NA	*
15	RF182	185	**	**	**	**
16	RF156	154	**	**	**	**
17	RF108	116	*	*	*	*
18	RF190	136	*	*	*	*
19	RF171	113	**	**	**	**
20	RF186	62	**	**	**	**
21	RF197	402	0	*	0	*
22	RF163	183	0	*	0	*
23	RF123	230	**	**	**	**
24	RF129	515	*	*	*	*
25	RF199	215	0	14	0	6.5

reversal in the sense of rotation, an interval of swimming which contributes to the movement of the cell could be observed.

Isolated motility defective mutants of *Hf. volcanii* could be categorised in different groups according to their swimming behaviour under the light microscope: i. the first distinct group of motility-defective mutants, isolation of which has been the primary goal of this project, are those which appear to have no motility under the light microscope. Colonies of these mutants usually possess properties of non-motile colonies on the swarm plates e.g. mutants RF113 and RF148 (Table 1); ii. The second group of motility defective mutants is very interesting: Their cultures at first consist of normal looking cells but their morphology alters rapidly. As the cultures grow older, the cells grow longer resembling noodles and spaghetti. In stationary phase, the majority of these cells appears like very long "snakes" under the phase contrast microscope. The pace and speed of this remarkable alteration seem to vary for each member of this group. It seems that cells pass through several different intermediate shapes to reach this "final" or "terminal" morphology. Through different stages of growth, there is always a minor population of cells that appears to be normal in their shape and swimming pattern. The number of these cells decreases as the culture grows older. The longer the cells get, the slower they become in their motility behaviour until eventually, the long snake-like cells locomote, if it could be possible at all, by oscillation. No spinning and/or rotation are normally observed in cells with this specific "final" morphology. These mutants produce colonies that possess the characteristics of non-motile mutants or, in most cases, form small and fuzzy swarms (e.g.

mutants RF129 and RF197). Reintroduction of these cultures into fresh medium results in cells of normal appearance which soon change into intermediate shapes and finally snake-like cells.

Hf. volcanii super-motile mutants that were isolated from the edge of swarms have a speed of rotation by far greater than the wild-type strain, although they still show the same swimming pattern. Swarms of these mutants in the swarm medium are similar to those of the wild-type but grow much faster.

Visualizing flagella:

Conventional procedures employed regularly to build up and stain bacterial flagella for light microscopy investigations (Colomé et al, 1986) failed in the case of *Hf. volcanii* flagella. Observing the cells under dark field microscope did not add more information to what had already been collected from phase contrast microscopy. Therefore, I tried to further characterize the motility-defective mutants by electron microscopy.

Electron microscopy:

Electron microscopy revealed further details about the cell morphology and flagella of

wild-type *Hf. volcanii* DS2 as well as its motility-defective mutants. *Hf. volcanii* DS2 cells grown in a liquid medium appear generally short in size and variable in shape e.g. oval, circular, triangular, rectangular, and other strange irregular forms which basically resemble potato chips (Figure 6). Cells picked from a several-week-old plate (Figure 7b) look basically the same as those grown in a fresh liquid medium. But when the liquid culture grows older and reaches stationary phase, a minor population of cells become elongated and more cylindrical (Figure 8). The major difference that has so far been noticed between these elongated cells and those of the short category, relates to flagellation. The short form cells are basically peritrichously flagellated. Every cell seems to possess up to several filaments that should physically come together in the form of a bundle to construct a helical structure. CW and CCW rotation of this helical structure pulls or pushes the cell back and forth and enables it to locomote in the environment. The older and elongated cells, however, are mono- or bi-polarly flagellated. The polarly situated flagellar bundle seems to be thicker and longer on the elongated cells compared to those of short cells. This phenomenon has been observed repeatedly with cultures being grown at 42 °C but has not been detected in cultures being grown under the same condition but at 37 °C.

Figure 6

Normal short-form flagellar filaments branching out peritrichously on several *Hf. volcanii* DS2 wild-type cells with different shapes

The samples were treated with glutaraldehyde and then negatively stained with 2% PTA. Flagellar filaments are shown with arrows. Magnification: (a) 28,000x; (b) 42,000x; (c) 28,000x; and (d) 42,000. Bar: (a) 0.5 μm; (b) 0.25 μm; (c) 0.5 μm; and (d) 0.25 μm.

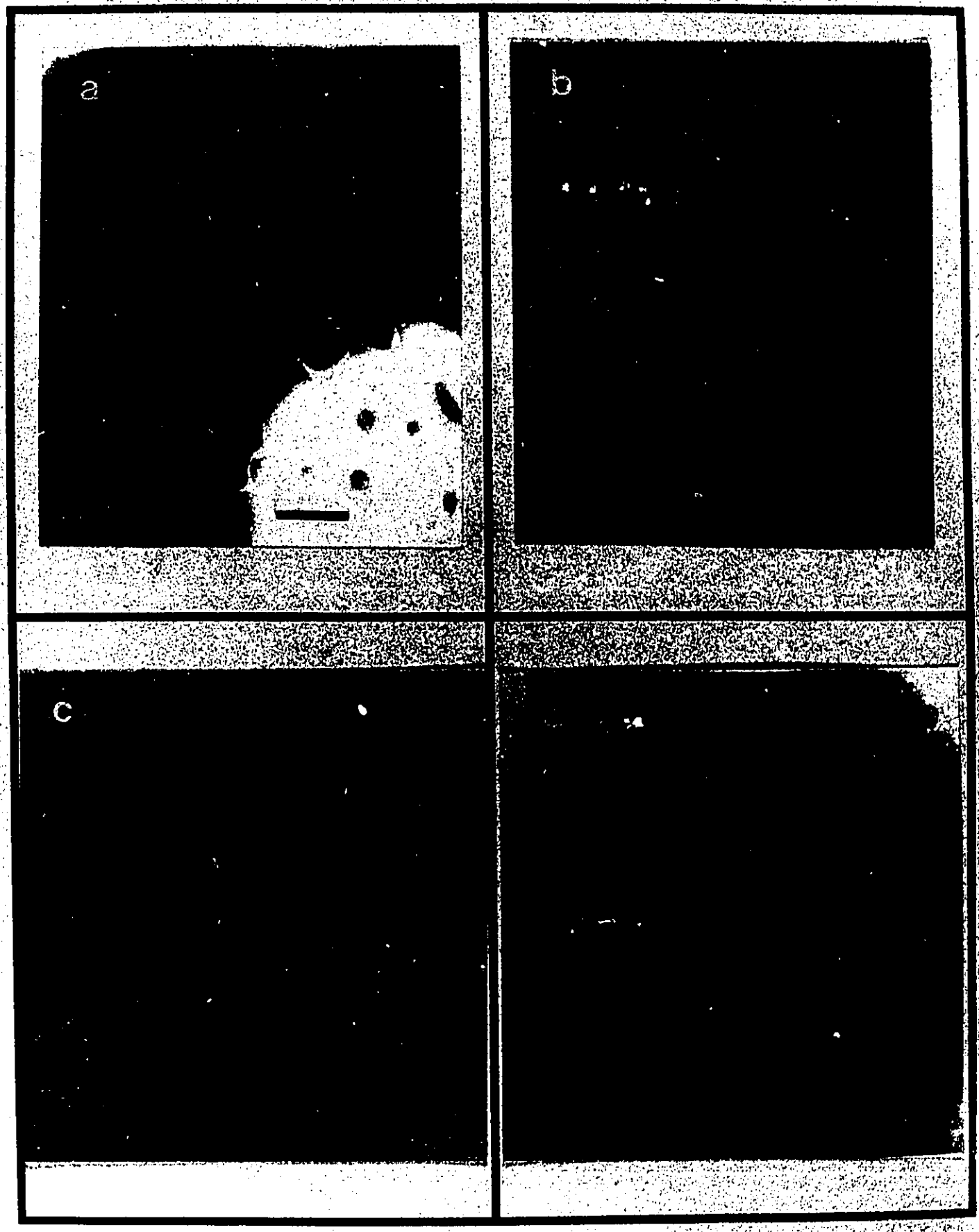

a
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Figure 7

Flagellated *Hf. volcanii* DS2 cells

a. Peritrichously flagellated *Hf. volcanii* DS2 cells grown in liquid medium with shaking. Cells were fixed and platinum-coated at an angle of 10° with rotation. Flagellar filaments are shown with arrows. Magnification: 14,000x. Bar: 1 µm.

b. Flagellated *Hf. volcanii* DS2 grown on a solid medium for several weeks. The arrow is pointing to a flagellar bundle. Cells were fixed and negatively stained with 2% PTA. Note that the flagellar filaments have formed a bundle. Magnification: 42,000x. Bar: 0.25 µm.

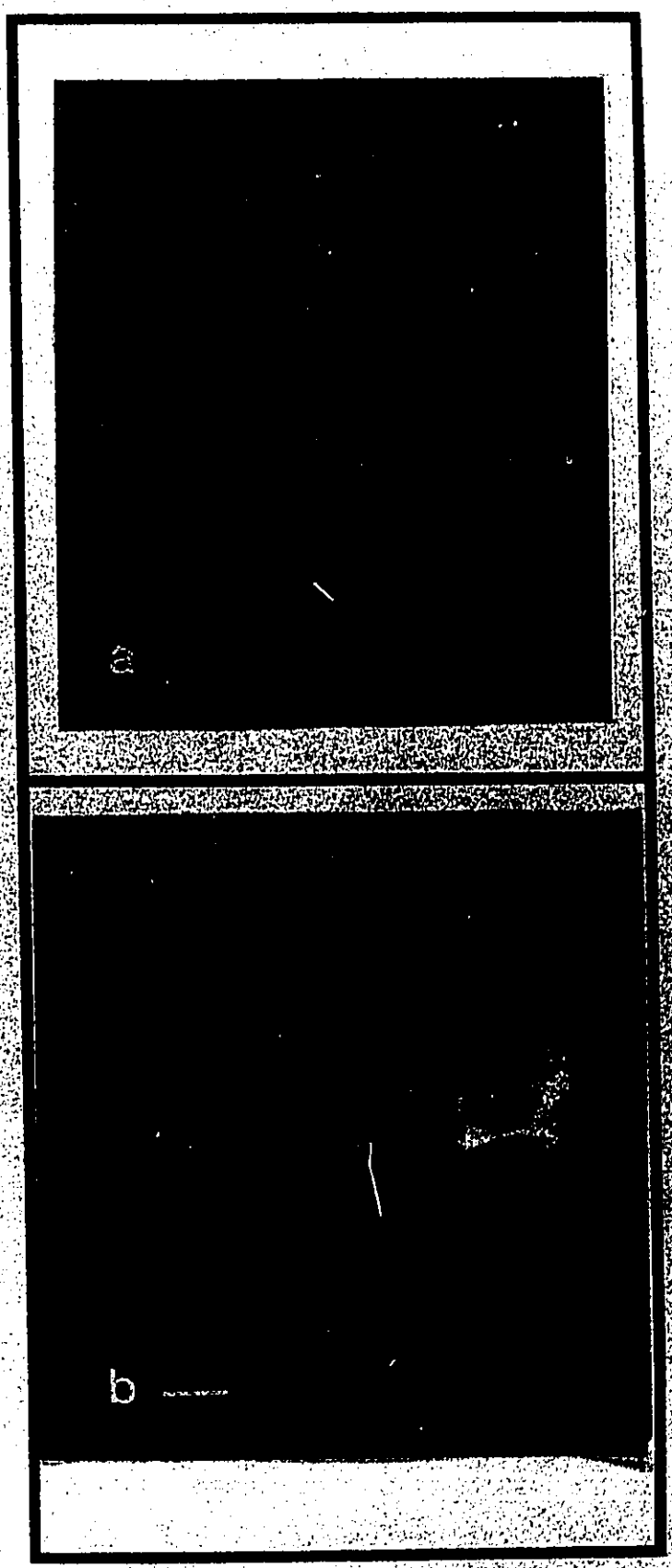
a
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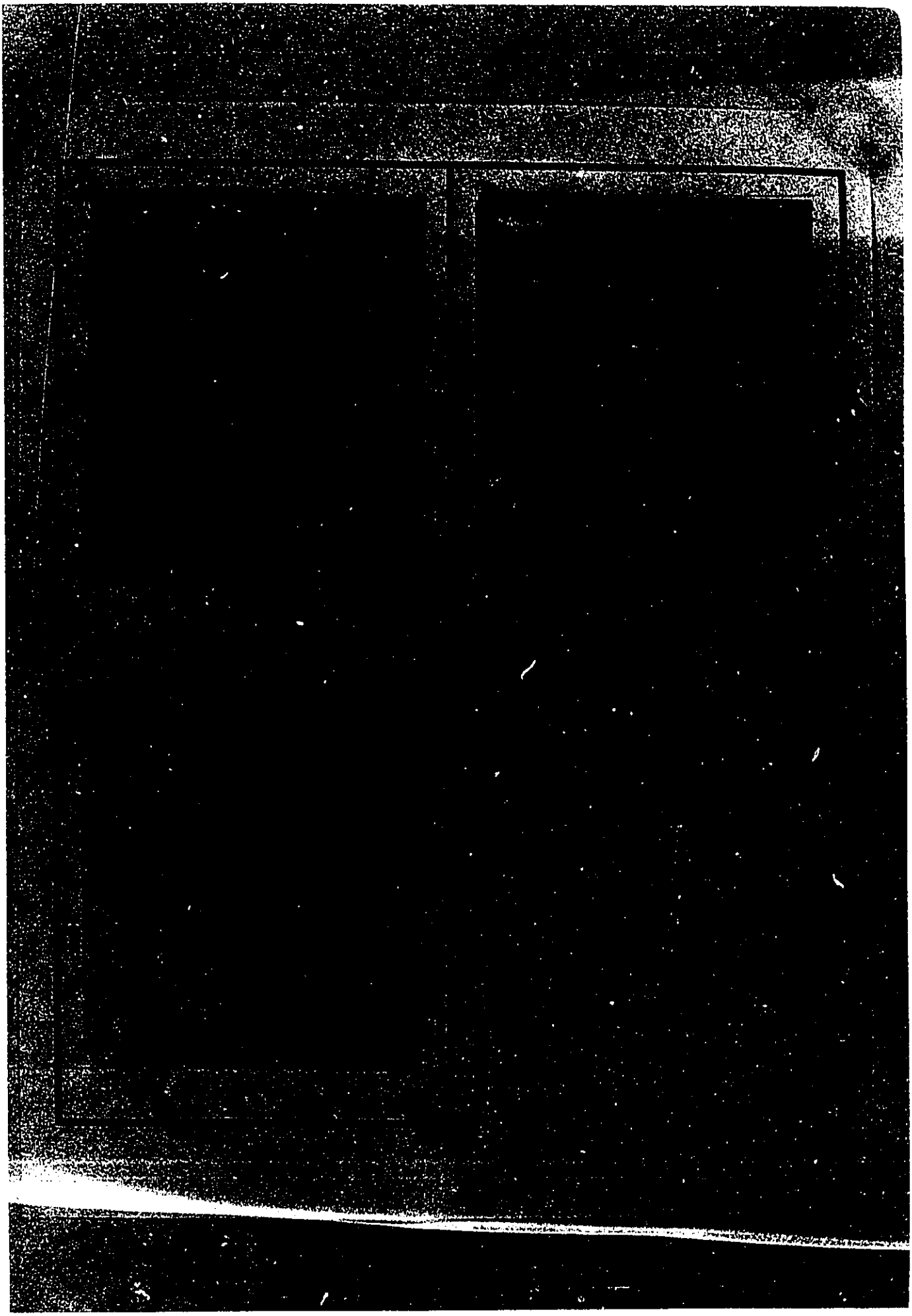
Figure 8

Polarly flagellated *Hf. volcanii* DS2 cells grown at 42 °C

Micrographs of elongated *Hf. volcanii* cells that are mono-polarly (a) or bi-polarly flagellated (b and c). Mono- and bi-polarly flagellated cells appeared when cultures were grown to stationary phase at 42 °C. Note that the flagellar filaments (shown with arrows) are much longer and thicker than the ones observed in peritrichously flagellated cells. Magnification: (a) 9,800x, (b) 21,000x, and (c) 28,000x. Bar: (a) 1 µm, (b) 0.5 µm, and (c) 0.5 µm.



On the grids prepared for electron microscopy with cells being grown in liquid medium with shaking, I frequently noticed that two or more cells were connected together by what seems to be cytoplasmic bridges (Figure 9). These bridges are the crucial structures for exchange of cellular (genetic) materials and bacterial mating (Rosenshine et al, 1989) but have not yet been described in broth-grown cultures. These cytoplasmic structures were only observed among the shorter ("younger") cells and have not yet been detected among rod-shape ("old") polarly flagellated cells.

Representatives of each group of isolated mutants were investigated by electron microscopy. A summary of the results obtained from these and other investigations for each mutant, and also for *Hf. volcanii* DS2, have been collected in individual tables in the Appendix.

The two non-motile mutants that were studied by electron microscopy, (RF113 and RF148), seemed to possess flagellar bundles of normal appearance (Figure 10) These cells were of short-form type and included irregular and long-form cells appearing as the culture grew older and approached stationary phase.

RF129 and RF197 mutants generally consist of short-form cells with normal flagellation in the early phase of growth (Figures 11 and 12). When the culture grows older, however, long snake-like cells begin to appear. The very long (spaghetti-like) cells dominate the culture along with many intermediate shapes (baseball bats, noodles, and ribbons) in the

stationary phase. The cells seem to be able to grow as long as tens of μm. Flagellar filaments in all intermediate and "final" forms of these mutants resemble normal short-form flagella of wild-type cells. Establishment of cytoplasmic bridges (mating) has been frequently noticed in these type of cells just like in the "younger" wild type *Hf. volcanii* cells (Figure 12b).

The super-motile mutants that were studied by electron microscopy (RF201 and RF202), appear generally short in cell shape (Figure 13). Every cell possesses dozens of flagellar filaments which are peritrichously distributed. The flagellar bundles seem to be on average longer and thicker than the flagellar bundles of normal short-form cells. Cultures of these mutants consist of short-form cells throughout the different phases of growth.

Are the motility defective mutants really *Hf. volcanii*?

To make sure that the motility defective mutants that I have isolated are indeed *Hf. volcanii*, genomic DNA was extracted from the mutants that were used in the described investigations. These DNA samples were digested with MluI and compared to the pattern that MluI-digested genomic DNA of *Hf. volcanii* DS2 produces when run on agarose gel. The results of this comparative test prove that the motility-defective mutants are indeed *Hf. volcanii* (data not shown).

Figure 9

Establishment of cytoplasmic bridges between short-form *Hf. volcanii* DS2 cells

Cytoplasmic bridges (large arrows) are established between two (b and c) or more (a) *Hf. volcanii* DS2 cells grown in liquid broth with shaking. In micrograph a, note the various cell shapes and also filaments (small arrows) that seem to be connecting cells together. These filaments may be necessary to connect cells together before they can establish cytoplasmic bridges. Cells were fixed with glutaraldehyde and then were negatively stained with 2% PTA (a and c) or 2% ammonium molybdate (b). Magnification: (a) 9,800, (b) 14,000, and (c) 28,000. Bars: (a) 1 µm, (b) 1 µm, and (c) 0.5 µm.

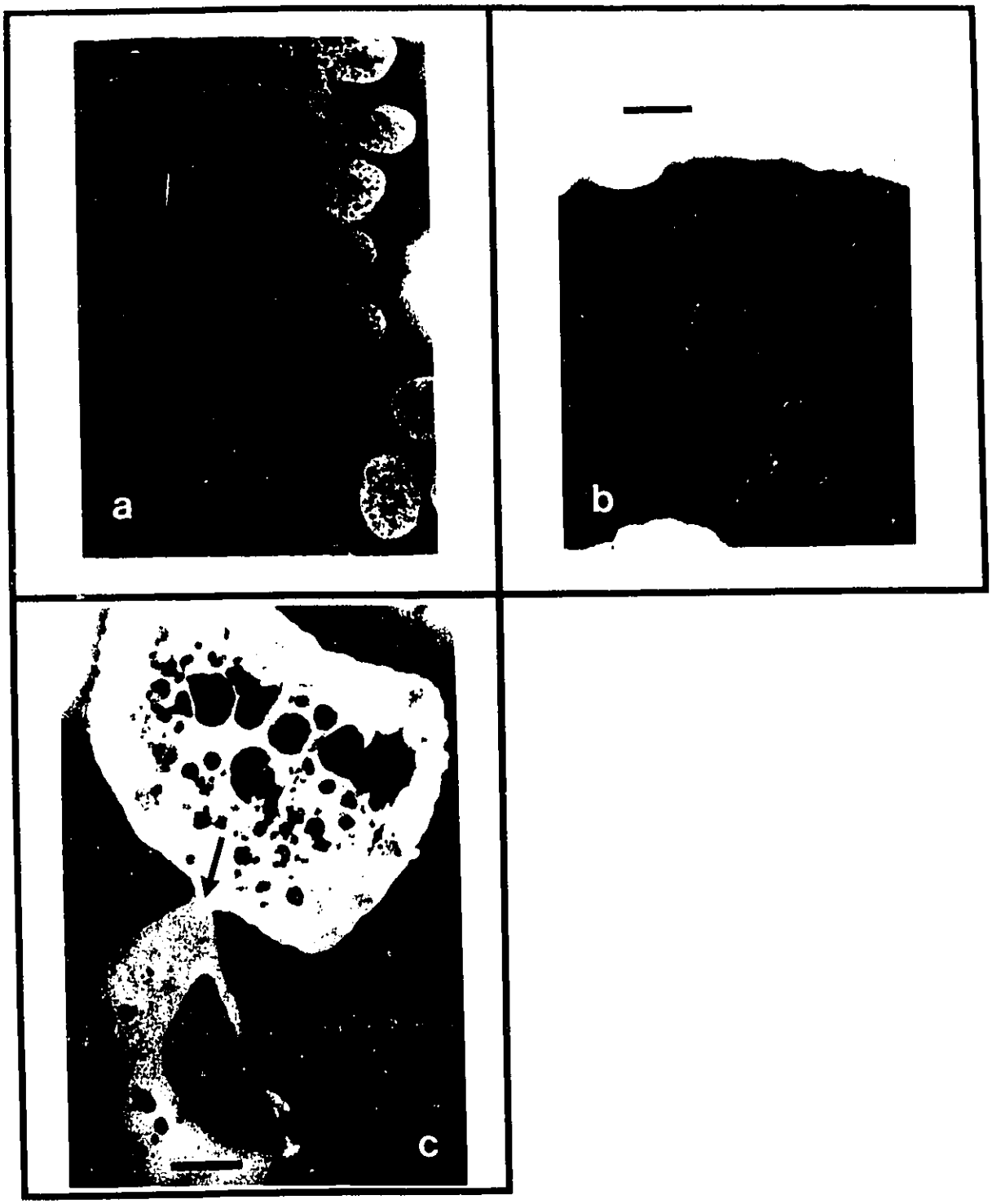

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Figure 10

Normally flagellated non-motile mutants of *Hf. volcanii*

Non-motile strain RF113 in short and long forms with normal looking flagellar filaments (small arrows) (a and b).

Non-motile strain RF148 in long form (c) and short form with normal looking flagellar bundles (small arrows) (d). Note the cytoplasmic bridge (large arrow) in micrograph d. These two strains form sharp and non-motile colonies on swarm plates and are non-motile under the light microscope. Magnification: (a) 28,000x, (b) 14,000x, (c) 28,000x, and 21,000x. Bars: (a) 0.5 µm, (b) 1 µm, (c) 0.5 µm, and (d) 0.5 µm.

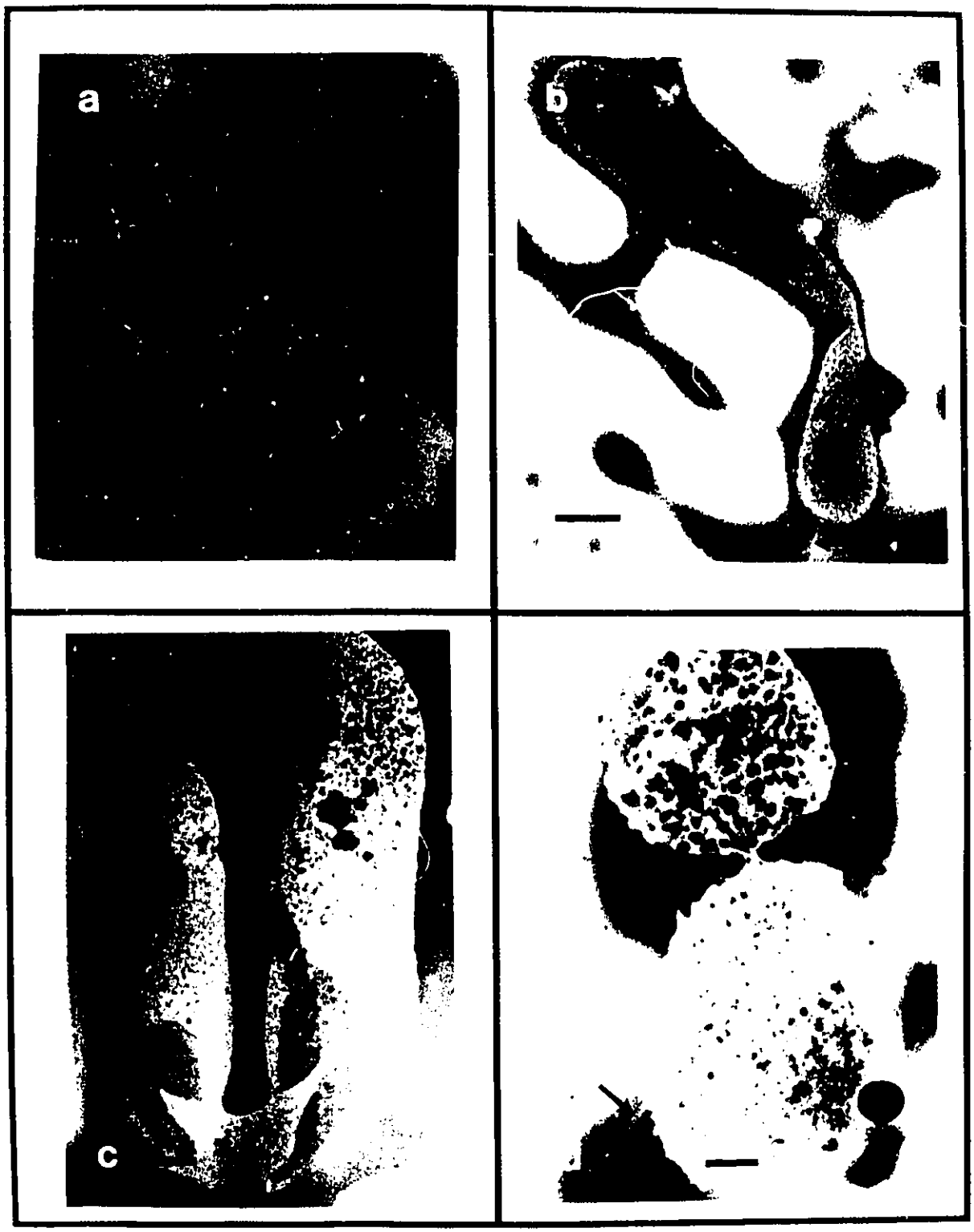
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Figure 11

***Hf. volcanii* mutants that produce long spaghetti-like cells**

Micrographs showing *Hf. volcanii* strain RF129 the culture of which consists of normal-looking short-form flagellated cells (a and b). But as the culture gets older, the cells stop dividing and form very long spaghetti-like cells (c and d). This mutant forms fuzzy small swarms on swarm plates. Short-form RF129 cells swim normally under the light microscope but the older and longer cells swim by oscillation. Note flagellar filaments (arrows) in cells of all sizes. Magnification: (a) 14,000x, (b) 14,000x, (c) 28,000x, and (d) 4,200x. Bars: (a) 1 μm, (b) 1 μm, (c) 0.5 μm, and (d) 2.5 μm.

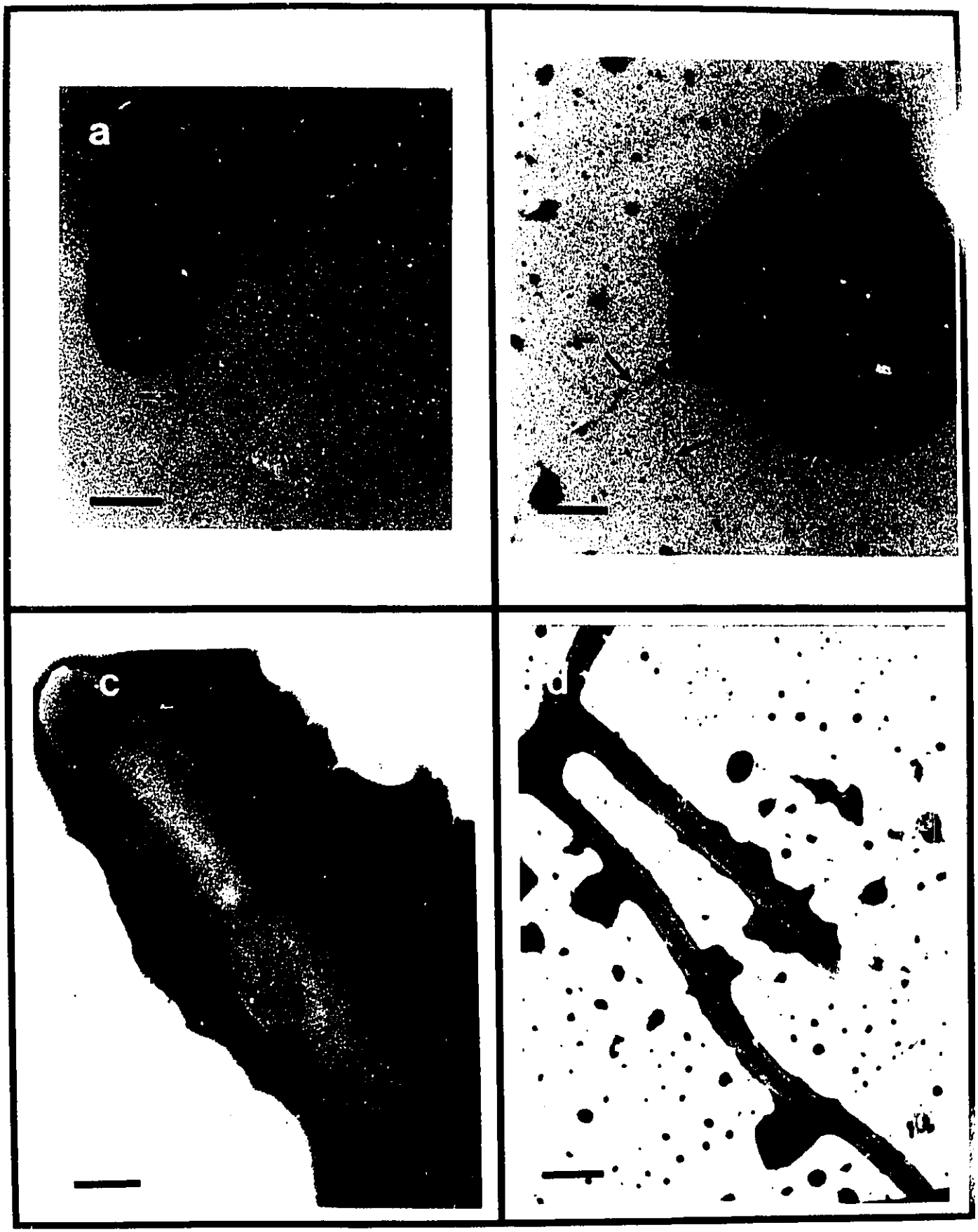
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Figure 12

Odd-looking intermediate morphology and cytoplasmic bridges in an *Hf. volcanii* mutant

Micrographs showing *Hf. volcanii* strain RF197 the culture of which consists of normal-looking short-form flagellated cells. But as the culture gets older, the cells stop dividing and begin an elongation process (a) that produces very odd-looking cells (c) before becoming spaghetti-like (b and d). This mutant forms small fuzzy swarms on the swarm plates. Short-form RF197 cells swim normally under the light microscope but old and long cells swim by oscillation. Note normal flagellar filaments (small arrows) in long cells (d) and establishment of cytoplasmic bridges (large arrows) between two long cells (b). Magnification: (a) 28,000x, (b) 14,000x, (c) 21,000x, and (d) 28,000x. Bars: (a) 0.5 μm, (b) 1 μm, (c) 0.5 μm, and (d) 0.5 μm.

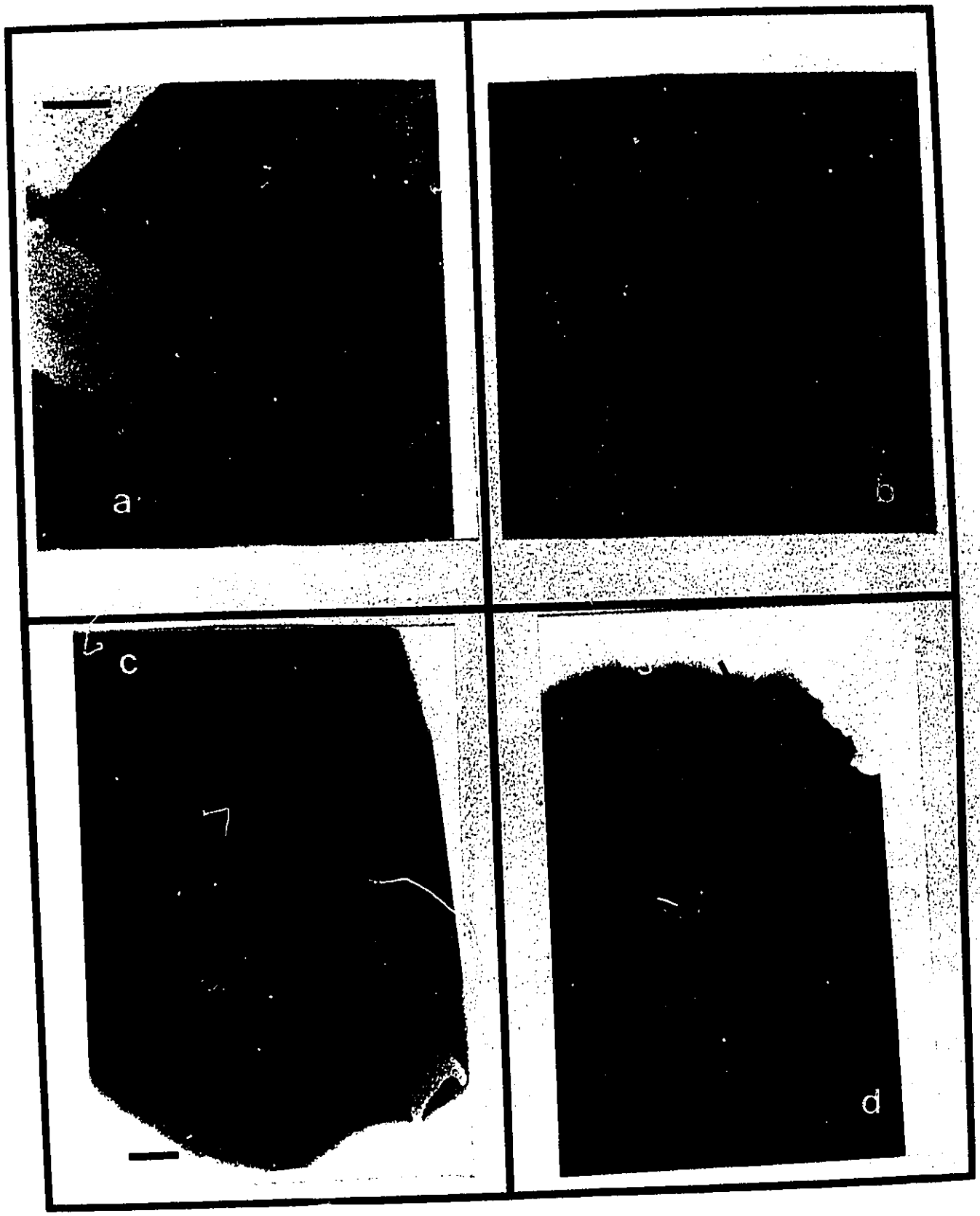
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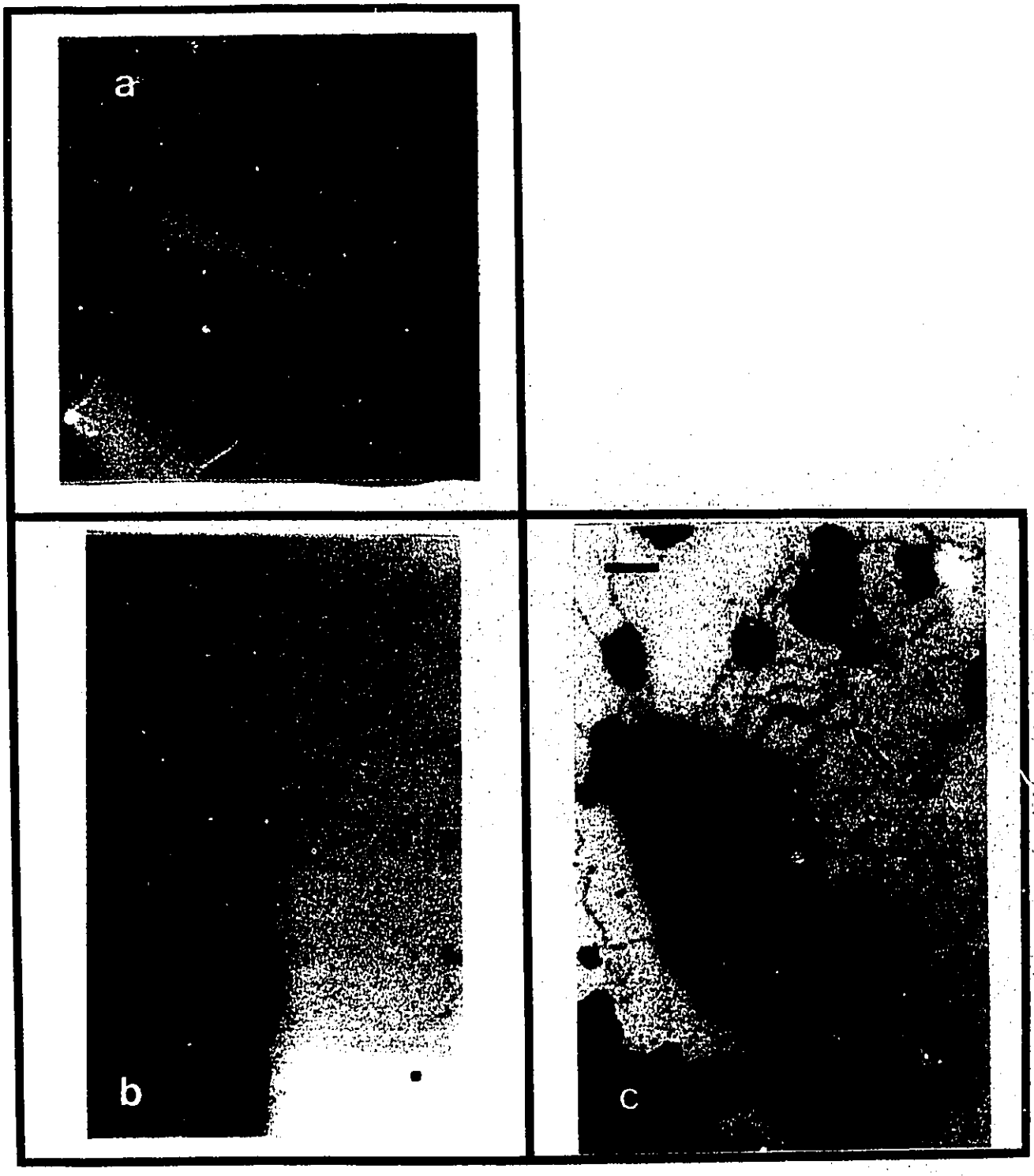
Figure 13

Flagellation in *Hf. volcanii* super-motile mutants

Micrographs showing *Hf. volcanii* strain RF202 super-motile cells. Note the longer length and the greater number of flagellar filaments (arrows) on each cell. These cells form normal swarms on swarm plates but their swarms grow much faster than wild-type. Under the light microscope, the super-motile mutants spin very fast in both directions and no spaghetti-like cells are observed when their cultures become older. Magnification: (a) 21,000x, (b) 10,500x, and (c) 21,000x. Bars: (a) 0.5 μm, (b) 1 μm, and (c) 0.5 μm.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***Hf. volcanii* DS2 genomic DNA shot-gun library:**

I first tried to construct the *Hf. volcanii* random (shot-gun) genomic DNA library using pWL101 (Lam and Doolittle, 1989), one of the most appropriate *Hf. volcanii-E. coli* shuttle vectors available at the time. *Hf. volcanii* total genomic DNA was digested with MluI and then ligated to MluI-linearized pWL101. This ligation was used to transform *E. coli* strain JM110 to obtain a filtered (Holmes et al, 1991) and amplified shot-gun library. The presence of pWL101 was verified by running plasmid or total genomic DNA extracted from randomly-picked transformed cells on agarose gel. The results revealed that most of the pWL101 plasmids which had been taken up by transformed cells had no insertions in them.

When several other suitable *Hf. volcanii-E. coli* shuttle vectors became available later, I tried to construct new shot-gun libraries using pLS46-D, pLS46-E, pLS46-2, and pLS46-8 (L.C. Schalkwyk, pers. commu.). I, later, found out that pLS46-D and pLS46-E have an extra MluI site outside their poly-cloning sites (data not shown). Therefore, I chose pLS46-8 from the latter pair as the vector of choice for it was smaller than its close relative, pLS46-2. The MluI-digested *Hf. volcanii* genomic DNA was ligated to MluI-linearized pLS46-8. This ligation was used to transform *E. coli* strain JM110 to overcome the restriction barrier and degradation of the DNA when it is introduced to *Hf. volcanii* (Holmes et al, 1991). Competent *E. coli* JM110 cells prepared with cold calcium chloride yielded only 10^2-10^3 transformants per μg of DNA. Therefore, I began to improve this

yield by using an electroporation technique to do the transformation. Electroporation methods increased the efficiency of transformation to $3x10^8$ transformants per µg of DNA which statistically covers the *Hf. volcanii* genome. Gel electrophoresis verification of the plasmids extracted randomly from the transformed members of *E. coli* strain JM110 showed that all examined transformed cells had been transformed with pLS46-8 containing at least one insertion (Figure 14a). Three different ligations were prepared and were filtered through *E. coli* strain JM110. The largest insertion is over 20 kbp in size (Figure 14b) probably equivalent to the largest fragment that is produced when *Hf. volcanii* genomic DNA is digested with MluI.

Genetic complementation of Motility-defective mutants:

I have attempted several rounds of transformation with representative members of different groups of motility-defective mutants using both sets of amplified libraries constructed with either pWL101 or pLS46-8 (results not shown). The transformation which is a well established procedure (Cline et al, 1989) was not been successful. Every transformation series included positive and negative controls. The positive control which is transforming competent cells with uncut plasmids such as pWL101, pWL102 (Lam and Doolittle, 1989) and pLS46-8 has given a transformation efficiency of 10^3-10^5 per µg of DNA. This is a lower than expected efficiency of transformation (Cline and Doolittle, 1992 and Cline et al, 1989). Further work is needed to improve transformation efficiency

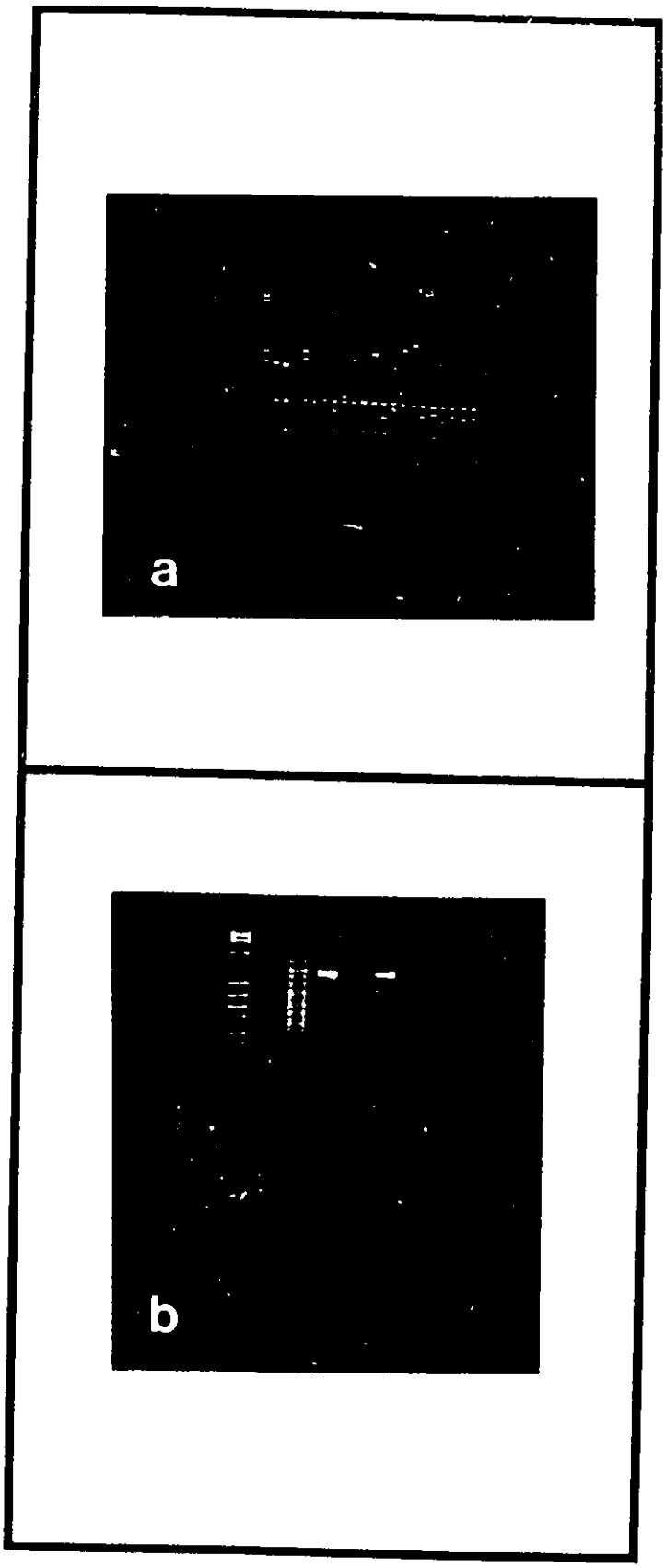
Figure 14

Representative insertions of *Hf. volcanii* DS2 shot-gun library and comparison of *Hf. volcanii* DS2 shotgun libraries with *Hf. volcanii* DS2 genomic DNA

a. Agarose gel showing 22 plasmid samples extracted from *E. coli* strain JM110 transformed with the *Hf. volcanii* DS2 shot-gun library. Plasmids were double-digested with HindIII and EcoRI. The larger similar bands are HindIII fragments and the smaller similar bands are EcoRI fragments of the plasmid vector pLS46-8. Note that there is at least one insertion of various sizes in addition to vector-derived fragments in each lane. In the first and last lanes, a mixture of λ DNA digested separately with BstEII, XbaI, and XhoI has been loaded as marker.

b. Agarose gel showing three different *Hf. volcanii* DS2 shot-gun libraries digested with MluI (lanes 4, 5, and 6 from left) run alongside two samples of *Hf. volcanii* DS2 genomic DNA also digested with MluI (lanes 2 and 3 from left) for comparison of patterns and distribution of insertions in pLS46-8 (the lighter bands in lanes 4, 5, and 6). In the left-most lane are markers as in (a) above.

Both gels had an agarose concentration of 0.8% and were run for 20 h at 20 V in TAE buffer.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to normal levels before another attempt can be made to complement the motility-defective mutants.

Discussion

Isolation of *Hf. volcanii* motility-defective mutants

Mutants play a very important role in the study of bacterial genetics. Production and/or isolation of mutants is normally the first step in a typical genetic project such as the present one which was originally designed to "clone and map genes involved in motility and taxis in *Hf. volcanii*". I began my project by serially transferring cells from the centers of swarms to new swarm plates in a bid to enrich *Hf. volcanii* cultures for spontaneous non-motile mutants and to select for cells that were not able to swim. Cells that have motility problems remain in the center of a swarm while all the other cells, naturally capable of swimming, swim outward. The speed of swarm formation was sharply reduced after a dozen such transfers which indicated the successfulness of the enrichment process. These colonies were grown and poured with hot swarm medium (containing 0.4% agar) to purify colonies of non-motile cells.

Meanwhile, independent cultures were being enriched for super-motile cells by repeatedly selecting cells from the edges of swarms and transferring them to new swarm plates. After 10-12 transfers of cells from the edges of swarms, the speed of swarm formation increased greatly (Figure 2). This was an indication that these cultures had been enriched for cells that had faster means of motility. These fast-growing colonies were used to

isolate the super motile mutants.

Why is colony appearance of the mutants different from wild-type?

The very preliminary indication that the isolated colonies were mutants came from their colony appearance. Therefore, the first question to address in characterizing the mutants was why the colony appearance of the mutants was different from the wild-type and how this related to their particular defect. There are three types of *Hf. volcanii* motility-defective mutants as judged by colony appearance:

i. sharp and non-motile solid colonies on swarm plates produced by mutants such as RF113 and RF148 (Figure 3): phase contrast microscopy confirmed that these mutants are non-motile and therefore, cannot swim outward to form swarms. These mutants are very stable and have a low rate of reversion (Table 1). The stability of these mutants may be due to a major genetic alteration such as a frameshift, a transpositional insertion, or a deletion. It may be difficult to complement mutants which have lost a large DNA fragment with shot-gun libraries because the average size of fragments in these libraries is 3-3.5 kbp. Cosmid clone complementation may be more useful in rescuing mutants with a large deletion of genomic DNA, but less information may be obtained from cosmid clone complementation due to the large size of cosmid clone inserts.

ii. fuzzy colonies produced by mutants such as RF129 and RF197 (Figure 4): these colonies begin to form swarms but stop before long and remain the same size. These colonies begin growing swarms because at early stages of growth, as documented by light microscopy and electron microscopy (Figures 11a and b), they consist of normally-flagellated short-form motile cells. These cells, however, become less and less motile as they stop dividing normally and continue elongating (Figures 11c and d). As a result, the small swarm stops growing and a steady increase in the overall mass of the population in the same area may contribute to the fuzzy appearance of the swarm. The size of these swarms depends on how fast, and on what percentage of, the bacterial population will stop dividing. In the two examined samples (RF129 and RF197), these two factors seem to differ notably.

iii. variable-sized swarms including non-motile colonies: The rate of reversion in this type of mutant is much higher than in type one, although the reversion rate for each of these mutants seems to vary notably from one to the other.

Some revertants of the mutants produce swarms with scalloped edges and dotted regions or swarms of variable sizes (Figure 5 a and b) formed as a result of frequent reversions within populations of mutants (Table 1). Although it is hard to predict, these mutants may be defective in genes that are not tightly regulated (such that they are sensitive to minor environmental fluctuations), or they may have a defect in a gene or in its regulatory mechanism which can be reversed frequently. Adverse conditions which could cause

motility defects such as lack of flagella production (Li et al, 1992 and Shi et al, 1992), and inappropriate changes in the production level of a regulatory factor (e.g. a trans-acting element that controls expression of other genes), may be responsible for the fluctuating behaviour of these kinds of unstable mutants.

Swarms produced by super-motile mutants are similar to wild-type swarms (Figure 2) except for the rate of dispersion which is much faster due to their greater number of flagellar filaments (Figure 13).

Characterization of *Hf. volcanii* and its mutants by electron microscopy

Electron microscopy revealed that the highly pleomorphic *Hf. volcanii* is normally peritrichously flagellated regardless of being grown in liquid broth or agar medium (Figure 6 and 7b). Between 4 and 10 flagellar filaments could be counted on *Hf. volcanii* cells. These flagellar filaments form a bundle due to mechanical and hydrodynamic forces and function as a cellular propeller (Figure 7b and 11a and b). The *Hf. volcanii* flagellar bundle could either be right-handed as in *Hb. halobium* (Alam and Oesterhelt, 1984) or left-handed as in *E. coli* (Macnab, 1987). In the first model, CW rotation of the bundle generates a force that pulls the cell and CCW rotation of the flagellar bundle pushes the cell (Alam and Oesterhelt, 1984). In *Hb. halobium*, which is polarly flagellated, the flagellar filaments do not fly apart when the sense of rotation changes. In *E. coli*, which

like *Hf. volcanii* is peritrichcusly flagellated, rotation of the flagellar bundle pushes the cell forward. When the sense of rotation changes, the bundle unwinds and the flagellar filaments fly apart. In these bacteria, changing of direction of swimming is achieved by tumbling (Macnab, 1987). No tumbling behaviour is apparent in *Hf. volcanii*.

In stationary phase cultures of *Hf. volcanii* that have been grown at 42 °C, a minor population of cells change their morphology and become elongated (Figure 8). This type of morphology has not been detected in young cultures or in cultures grown at 37 °C. Therefore, it is possible that this elongation of cells is temperature-dependent and that the lower temperature is not permissive for it. Flagellation in these elongated cells is distinctively different than the flagellation in younger short-form cells. The elongated cells are much like *Hb. halobium* and are mono- or bi-polarly flagellated. This experiment was repeated several times to eliminate the chances of contamination, a possibility which is still not completely ruled out. *Hb. halobium* (polarly flagellated) requires a higher amount of salt in its medium (Stoeckenius and Rowen, 1967), therefore it is unlikely that it could grow in *Hf. volcanii* medium which contains a much lower amount of salt. Since the proportion of polarly-flagellated cells was very low, several attempts to verify their genomic DNA pattern failed. If contamination of cultures is not responsible for this observation and flagellation is truly different in short-form and elongated cells, *Hf. volcanii* should have a mechanism responsible for repositioning its flagellar filaments as the cell grows in length and becomes cylindrical. The simplest explanation may be an addition of new cell wall material at the equator (Jacob et al, 1963) of the peritrichously-

flagellated cell which elongates the cell and at the same time moves the flagellar filaments away from the equator to the poles. The polar flagellar filaments are seen to emanate from the polar hemispheres, not the apex (Figure 8). It is also very obvious that the flagellar filaments in the lophotrichously flagellated cells are longer and thicker. This may indicate that these filaments are different from the ones in younger short-form cells; they may be constructed differently or from different monomers which makes the issue more complex and ambiguous. Therefore, it should not be surprising if more than one kind of flagellin protein is found in *Hf. volcanii*. Environmental signals may be important in triggering synthesis and/or assembly of different flagellar proteins (Li et al, 1992 and Shi et al, 1992). Repeating this experiment with cells grown at yet higher temperatures, e.g. 45, or even 50 ^{o}C, may provide information that will address this issue. In *Hb. halobium*, the cells are mono-polarly flagellated in young cultures but as the culture approaches stationary phase, the cells become bi-polarly flagellated (Alam and Oesterhelt, 1984). This may be important in the motility of cells when food is decreasing and better swimming ability is a privilege. Although one cannot distinguish between short-form and elongated cells under the light microscope, the swimming pattern in polarly flagellated *Hf. volcanii* might resemble the swimming pattern in *Hb. halobium*.

Flagellation in non-motile mutants:

Mutants RF113 and RF148 that produce non-motile colonies on swarm medium and are

non-motile under the phase contrast microscope, seem to possess normal flagellar filaments (Figure 10a). The cause of the motility problems in these mutants cannot be detected by techniques employed in this project. Their flagellar filaments can, for instance, be paralysed or they may have structural defects. These problems could be studied by using methods such as laser dark-field microscopy (Kudo et al, 1990) or sensitive video techniques for recording flagellar filament movement in intact cells (Alam and Oesterhelt, 1984, and Block et al, 1991). In the stationary phase, some of these cells stop undergoing cell division and form noodles or spaghetti-like cells (Figure 10b and c). Flagellation in noodles and spaghetti-like cells seems to be the same as in short-form cells (Figure 10b, 11c and 12d) except for the number of the filaments which decreases compared to short-form cells (Figure 11a and b).

Flagellation in super-motile mutants:

Super-motile mutants RF201 and RF202 are always of short-form and would not grow to be very long in stationary phase cultures. These mutants possess flagellar filaments several times greater in number compared to wild-type *Hf. volcanii* (Figure 13). There are over 30 flagellar filaments visible in one of these cells (Figure 13a). Figure 13b and 13c provide good evidence of flagellar filaments forming a bundle. The greater number of filaments may be due to mutations in genes responsible for regulating flagellar filament assembly, or amplification and/or over expression of flagellin genes which result in the

production of more flagellin. Most of the time there is some loose flagellar filaments in these cultures. Some of these loose filaments could be seen in Figure 13b and c.

Mating in *Hf. volcanii*:

As a side observation of characterizing *Hf. volcanii*, I frequently found the cells to have established cytoplasmic bridges that are necessary structures for mating (Figure 9, 10d and 12b). Mating in halobacteria has been reported on solid support but not in liquid cultures with shaking (Mevarech and Werczberger, 1985 and Rosenshine et al, 1989). I have, however, not been able to find any evidence that the polarly flagellated cells can mate. Mating has also been observed among filamentous cells (Figure 12b) and between filamentous and short-form cells (data not shown). This is an apparent similarity between normal short-form *Hf. volcanii* cells and the spaghetti-like mutants.

Organization of genes involved in motility

Out of tens of genes involved in motility and taxis only the flagellin genes (Gerl and Sumper, 1988) and the *sop* (sensory rhodopsin) gene (Blanck et al, 1989) from *Hb. halobium* have been cloned and sequenced. Therefore, there are many other genes involved in motility and taxis to be cloned and studied. The mutants isolated in this

project are valuable mutants that could be used to identify some of these genes in *Hf. volcanii*. These genes may be organized in functionally ordered operons and clusters such as in eubacteria. The two flagellin gene operons in *Halobacterium* sp. GRB have been mapped close to each other on the chromosome (R.L. Charlebois pers. commu.). Early observation shows that the two flagellin operons can recombine.

Genes involved in motility in *E. coli* (Macnab, 1987) and *C. crescentus* (Ohta et al, 1991) are organized in a regulatory hierarchy. At the top of this hierarchy in *E. coli*, there is a master operon including only *flaI* and *flbB* genes. Expression of genes in the lower levels in the hierarchy requires the expression of this master operon. Expression of *flaI* and *flbB* is regulated by a complex of cAMP and CAP (catabolic activator protein) which binds to the *cfs* (constitutive for flagellar synthesis) sequence upstream in the hierarchy. Presence of sugars such as D-glucose as the carbon source (Adler and Templeton, 1967) in the growth medium strongly represses flagellum synthesis. Addition of cAMP reverses this catabolic repression. There is evidence that *flaI* and *flbB* genes are the only motility genes that are controlled by catabolic repression (Parkinson and Hazelbauer, 1983).

Cell-cycle defective mutants

An unexpected result of this project was the isolation of *Hf. volcanii* mutants defective in their cell cycle. These mutants have normal flagellar filaments which form flagellar

bundles (Figure 11a and b) when the cells are in short-form stage. But as the culture grows older, the cells stop dividing and grow until they form very long cells which are tens of μm long. The issue of cell cycle and its probable coupling with motility (Yu and Shapiro, 1992) is a very complicated issue in which many genes are involved (Ishihama, 1991 and Nishimura et al, 1991). Defects in many of these genes may result in cell cycle and cell division defects (Donachie and Robinson, 1987) with repercussions on motility. The cell cycle is very tightly controlled by a cascade of genetic and biochemical events to ensure the fidelity of the process. Control mechanisms inhibit cells that have made serious mistakes from entering the next step of the cell cycle (Helmstetter et al, 1979). This inhibition will result in abnormal cells.

Cell growth and division:

Synthetic activities during cellular growth increase the cellular mass to a critical volume (Helmstetter, 1987) which instigates a cascade of genetic and biochemical events that triggers chromosome replication and cell division (Donachie, 1968). There seems to be a simple strain-specific relationship between mass (volume) and growth rate which governs the cell size at division time (Donachie and Robinson, 1987). In *E. coli*, cellular growth seems to occur only in the length of the cell whereas cell width does not change (Marr et al, 1966). Electron microscopy, however, shows that cellular width in *Hf. volcanii* mutants vary greatly (Figure 12c) which makes some of these cells look like

noodles and/or baseball bats. These cells also show variation in their thickness along the cellular body. Scanning electron microscopy (data obtained after thesis submission, not shown here) clearly shows that in the noodles and spaghetti-like cells, some parts are extremely flat and some parts are swollen like a small ball. This may contribute to an abnormal distribution of genetic materials and chemical factors, dilution (Pritchard et al, 1969) and/or accumulation (Sclafani and Wechsler, 1981) of which may be involved in a trigger system that controls cell growth and cell division. It would be very interesting to know how this mechanism functions. Investigations in this area, using mutants isolated in this study, may also lead to findings about genes and mechanisms that control cell shape and pleomorphism in *Hf. volcanii*.

Genes involved in the cell cycle:

In the past two decades, many attempts have focused on clarifying different aspects of the cascade of events that are orchestrated so remarkably to govern the cell cycle. Nevertheless, many aspects of the cell cycle still remain unknown. With recent advances in molecular biology and molecular genetics, many different mutants defective in cell cycle and cell division have been isolated and investigated and hundreds of genes have been cloned (Nishimara et al, 1991, Ishihama, 1991 and Suzuki, 1991). These mutants have produced valuable information about the cell cycle and cell division which seem to be conserved, to some degree, among eubacteria (Ogasawara et al, 1991).

Evidence for coupling of flagellar genes to genes involved in the cell cycle:

Early evidence of coupling of flagellar production with the cell cycle was found in Silverman and Simon's work (1977). Now, by studying cell cycle and motility mutants, more and more evidence is being found (Ramakrishnan et al, 1991, Dingwall et al, 1992, Shi et al, 1992, and Yu and Shapiro, 1992) suggesting that products of genes such as *dnaK* and *dnaJ* that are important in cell division and DNA synthesis (Gross et al, 1990) are also required for flagellum synthesis (Shi et al, 1992). Mutation in these genes results in inhibition of flagellum synthesis and production of non-flagellated mutants. Mutation in DnaK has a more severe effect on flagellum synthesis than a decrease in cAMP-CAP complex, by decreasing the expression of *flaI*, one of the genes in the master operon of the flagellar hierarchy. This means that flagellar gene expression is both under the control of a cell cycle signal and nutritional conditions. Mutations in genes that are located at the top or near the top of the flagellum synthesis hierarchy such as those in the *flbO* locus and also the *flaS* gene in *C. crescentus* result in non-motile cells that are defective in cell division (Yu and Shapiro, 1992 and Dingwall et al, 1992). Genetic complementation of *flaS* mutants restores both motility and normal cell division (Dingwall et al, 1992). These findings indicate that motility machinery and cell cycle are related to some degree. Coupling of flagellar gene expression with cell division in a functional genetic hierarchy is a logical means of avoiding costly production of flagella before necessary steps in cell growth and cell division have been correctly completed. This mechanism may also be functioning as a check point where the bacterium verifies that all steps such as DNA

replication have been successfully completed before entering the next step. This may be the reason why some of the cell division mutants are either non-motile or have motility problems as well.

Importance of *Hf. volcanii* mutants in cell cycle studies:

The cell cycle and cell division-related areas in archaebacteria have remained untouched and thus our knowledge about the cell cycle, and about growth and cell division mechanisms and their controls in archaebacteria is so small. The cell-cycle-defective mutants that have been isolated in this project are very valuable since they are among the very first halophilic mutants defective in the cell cycle. The importance of *Hf. volcanii* mutants defective in cell division comes from the fact that investigation of these mutants will provide exciting information about cell cycle and cell division in archaebacteria (halobacteria). This information may or may not support the conservation of cis- and trans-acting elements that are involved in Gram-positive and Gram-negative eubacterial cell cycle and cell division (Ogasawara et al, 1991, Ogasawara et al, 1985, Fujita et al, 1989, Fujita et al, 1990 and Calcutt and Schmidt, 1992). Comparative studies between the major lineages of life will open new avenues in finding out about the cell cycle mechanism and its genetics in the organism that served as their common ancestor and also about the evolution of the reproductive system in bacteria.

Determining the relationships between cellular mass, DNA content and chromosome copy number per cell of the cell-cycle mutants will provide very useful information in understanding *Hf. volcanii* growth mechanisms (von Meyenburg and Hansen, 1987, and Donachie and Robinson, 1987). This information might demonstrate how cellular mass can function as the trigger (Helmstetter, 1987) of chromosome replication initiation and as the trigger of cell division in halobacteria. Information obtained from characterization of these mutants will be very useful in comparative evolutionary studies which will shed more light on the mechanism of cell division and cell cycle in the ancestor of procaryotes and its divergence in the two domains of Archaea and Bacteria.

What is wrong with *Hf. volcanii* mutants defective in cell division?

Hf. volcanii mutants defective in cell division do not divide normally and continue growing until they form filamentous cells. The filamentous cells may be blocked in normal cell division due to:

A. Cellular mass increase is not able to trigger initiation of chromosome replication: this may be caused by a defect in a signalling process that is responsible for initiation of chromosome replication. This signal is produced when the cell reaches a critical size and volume and functions as the trigger of chromosome replication initiation (Helmstetter, 1987). It is important to investigate growth characteristics of *Hf. volcanii* to find out about

its critical cellular volume and mass.

B. Chromosome replication cannot initiate: mutation in cis- and trans-acting elements involved in chromosome replication may result in a defect in the initiation process, even though the trigger mechanism which was discussed above may function normally. Estimating the number of oriC and calculating the oriC/mass ratio in the filamentous cells will indicate if the filamentous cells are impaired in this process. Therefore, it is necessary to first find out the specific oriC/mass ratio in wild-type *Hf. volcanii*. If the filamentous cells have only a single oriC per cell, either the trigger for initiation or the mechanism of initiation of replication has become defective and the cells continue growing without initiating chromosome replication.

C. Initiation of chromosome replication is taking place but it is not completed: perturbation of chromosome replication in *E. coli* inhibits the cells from continuing the cell cycle process through activation of the SOS response (Walker, 1987 and Cole, 1983) which is responsible for DNA repair (Donachie and Robinson, 1987). This causes the cell to grow without being able to divide. This issue can be addressed in the *Hf. volcanii* mutants by finding out how many oriC's are present in a filamentous cell and comparing that to the oriC/mass ratio in normal cells. Measuring the DNA content or specific staining of the cellular DNA of filamentous cells will show if there are enough copies of the chromosome for the existing cellular size. Presence of enough oriC's but less DNA than expected will be an indication of normal rounds of chromosome replication initiation

but inhibition of their completion.

D. Newly produced nucleoids do not undergo segregation: some of the cell division mutants may be normal in chromosome replication but are defective in separation and repositioning of the newly synthesised chromosomes. Inhibition of nucleoid segregation may cause an inhibition of the cell division process (Hiraga, 1991). DNA staining will show if the completely replicated chromosomes are not able to segregate and therefore, accumulate at the same point or are randomly scattered throughout the cell.

E. Septum initiation and formation is impaired: segregation and repositioning of nucleoids can be checked by specific DNA staining. If the newly-synthesized chromosomes have been correctly positioned in the centers of the prospective daughter cells but the process does not continue, it can be realized that the initiation of septum formation has been somehow affected.

In this study, the data obtained so far indicates that inoculation of filamentous cells into fresh broth, under the same growth conditions, gives rise to a new normal-looking culture at least during the early period of growth. This may have more than one explanation: i. a number of short normal cells are always present in the culture where the dominant morphology is filamentous. These short-form cells may be responsible for the growth of normal-looking new cultures. ii. some of these mutants that are not able to undergo cell division are conditional mutants that act differently at different stages of population

growth. These mutants may regain their conditional ability to undergo a limited number of cell divisions due to environmental signals.

Construction of shot-gun libraries:

Many attempts were made to construct shotgun libraries using pWL101 (Lam and Doolittle, 1989), based on the *Hf. volcanii* pHV2 replicon (Charlebois et al, 1987) which has been widely used in transformation of *Hf. volcanii* (Cline et al, 1989). pWL101 has a unique MluI site and genes for mevinolin and for ampicillin resistance. I was, however, unable to construct good libraries with pWL101 and many of the examined clones did not carry genomic fragments (data not shown). MluI is the preferred cloning enzyme because it cuts *Hf. volcanii* DNA randomly and its fragments are well-characterized from the genome mapping project (Charlebois et al, 1989).

pLS vectors (L.C. Schalkwyk, pers. commu.) are generally smaller in size which is an important factor in transformation efficiency and also carry a multi-cloning site. pLS46-E and -D also carry mev and ampicillin resistance genes. I found out that they have an extra MluI site in their mev gene region (data not shown). This extra site should be eliminated before these vectors become truly useful for MluI-cloning. Thus, I chose pLS46-8 which has one MluI site in its multicloning site but which carries the trimethoprim-resistance gene (tmp^r) instead of mev^r. pLS46-8 is smaller than a similar construct called pLS46-2.

Attempts were quite successful and three independent *Hf. volcanii* genomic DNA shot-gun libraries were constructed. These libraries have successfully been amplified through *E. coli* strain JM110. Their insert distribution patterns compared to MluI-digested *Hf. volcanii* genomic DNA, suggests that they have a good distribution of insert sizes (Figure 14 a and b). Transformation of *E. coli* JM110 competent cells prepared by treating them with cold calcium chloride (Sambrook et al, 1989) gave very low efficiency but a higher efficiency of transformation (10^8 transformants per μg of DNA) was obtained using electroporation techniques (Calvin and Hanawalt, 1988).

Transformation and genetic complementation of mutants:

Several attempts have been made to complement the motility-defective mutants, but no results are yet available. The efficiency of transformation with uncut plasmids as positive control is far lower than reported (Cline et al, 1989). Complementation of cell-cycle mutants may be particularly difficult because of their huge cellular mass which may make them more sensitive to critical details of the transformation procedure. Several attempts to transform these mutants have been unsuccessful because the cells tend to lyse more rapidly. Lysis of cells in PEG-mediated transformation (Cline et al, 1989) causes DNA precipitation which lowers the efficiency of transformation greatly (S.W. Cline, pers. commu.). An effort should nevertheless be made, since complementation of these mutants may result in cloning cell-cycle and cell-division genes in *Hf. volcanii*. The efficiency of

transformation can be improved by using competent cells when they are younger and less susceptible to lysis. Given that three amplified shot-gun libraries have been constructed and some of the isolated mutants have been characterized, we must work at improving the efficiency of transformation to complement the mutants and to clone genes that are responsible for motility and cell cycle control in *Hf. volcanii*. Cloning and mapping more genes involved in motility and cell cycle will reveal the organization of related genes in halobacteria and their mode of interaction and regulation. The most interesting clones will be those that can restore both motility and a normal cell cycle. Studying these clones will help to understand the motility and cell cycle gene organization and the relationship between cell cycle and motility in archaebacteria. Cloned genes can then be used to probe motility and cell cycle genes in other archaebacteria. Sequencing and comparison of these genes with homologous genes in eubacteria will provide interesting information about cell cycle and motility in their ancestor and also about the evolution of these genes. Finding conserved sequences in archaebacterial motility and cell cycle genes will probably lead to the conclusion that the two lineages of living prokaryotic organisms have inherited these properties from their common ancestor. If no similarities are found, it would be conclude that these properties have evolved independently and that the organism that served as the ancestor of eubacteria and archaebacteria was perhaps not motile.

Appendix

Detailed observations of mutants isolated in this study:

In this Appendix, I have tabulated a summary of observations for each of my mutants and for the wild type *Hf. volcanii* DS2.

Phase contrast microscopy was performed on cultures at three stages of growth: early-log (≈ 21h), mid-log (≈ 45h), and stationary (≈ 72h). Cells, pelleted by a bench-top clinical centrifuge and resuspended in *Hf. volcanii* broth containing no tryptone or yeast extract (salt solution), were also examined to see the effect of centrifugation on motile cells. Spinning at low speed did not seem to affect motility characteristics.

It is very important to note that although noodles and filamentous morphologies have been observed for many of the mutants, the time, the rate and the percentages of conversion of short-form cells to other morphologies for every sample is notably different.

In general, the isolated mutants all fall in the category of non-motile cells, but since some classes of motility mutants are able to swim, e.g. Che-, I have tried to categorize each mutant as either motile or non-motile.

Strain: *Haloferax volcanii* DS2

Growth Condition Time Temp.	Culture Appearance	Dominant Morphology	Other Morphology	Motility Description
20h 38 °C	light turbid	short	elongated, filaments	They are motile.
48h 38 °C	turbid	short	-	motile
72h 38 °C	pale pink	short	-	motile
Colony appearance on swarm plate: They form swarms which can grow as large as the plates.				
Colony appearance of the revertants on swarm plate: N/A				
Microscopic observation of the revertants: N/A				
Electron microscopy: Motile, pleomorphic, peritrichously flagellated, cytoplasmic bridges between cells, old elongated cells mono- or bipolarly flagellated.				
Category: Motile				

Strain: RF108

Growth Condition Time Temp.	Culture Appearance	Dominant Morphology	Other Morphology	Motility Description
20h 38 °C	slight turbid	short	elongated, noodles, filaments	Motile. No spinning and no swimming. Most of them seem to swim straight.
48h 38 °C	turbid	elongated	short, noodles, filaments	Short cells spin; filaments oscillate.
72h 38 °C	pale pink	filaments	short, elongated	Motile. The shorter the cells, the faster.
Colony appearance on swarm plate: Small swarms. All colonies form swarms but not as large as the wild type.				
Colony appearance of the revertants on swarm plate: N/D				
Microscopic observation of the revertants: N/D				
Category: Motile				

Strain: RF113

Growth Condition Time Temp.	Culture Appearance	Dominant Morphology	Other Morphology	Motility Description
21h 42 °C	slight turbid	short	elongated, noodles, filaments	Non motile
45h 42 °C	turing pink	short	elongated, noodles, filaments	Non motile
72h 42 °C	pink	short	elongated	No swimming or spinning but it seems that some jiggle where they are.
Colony appearance on swarm plate: Two swarms out of 360.				
Colony appearance of the revertants on swarm plate: N/D				
Microscopic observation of the revertants: Short and elongated cells with normal-looking flagella.				
Category: Non motile				

Strain: RF115

Growth Condition Time Temp.	Culture Appearance	Dominant Morphology	Other Morphology	Motility Description
21h 42 °C	slight turbid	short	elongated, filaments	Non-motile, no spinning, no swimming. Some seem to be slightly motile.
45h 42 °C	turning pink	short	elongated, filaments	Non-motile. No spinning, no swimming. Some cells jiggle.
72h 42 °C	pale pink	short	elongated, filaments	Motile.
Colony appearance on swarm plate: Non-motile.				
Colony appearance of the revertants on swarm plate: N/D				
Microscopic observation of the revertants: N/D				
Category: Non motile to Motile				

Strain: RF120

Growth Condition Time Temp.	Culture Appearance	Dominant Morphology	Other Morphology	Motility Description
20h 38 °C	slight turbid	short	-	Motile. No spinning and no swimming.
48h 38 °C	turbid	short	elongated, noodles, filaments	No swimming or spinning. But they are slightly motile.
72h 38 °C	pale pink	elongated	short, noodles, filaments	No spinning or swimming but some are motile. The noodles and filaments oscillate.
Colony appearance on swarm plate: Small swarms.				
Colony appearance of the revertants on swarm plate: N/D				
Microscopic observation of the revertants: N/D				
Category: Motile				

Strain: RF123

Growth Condition Time Temp.	Culture Appearance	Dominant Morphology	Other Morphology	Motility Description
21h 42 °C	slight turbid	short	elongated	They spin and swim. The shorter they are, the faster they swim.
45h 42 °C	turbid	short, elongated	noodles, filaments	Swim in different directions. Filaments oscillate.
72h 42 °C	pale pink	short, elongated	noodles, filaments	Motile.
Colony appearance on swarm plate: Large pigmented swarms with circles.				
Colony appearance of the revertants on swarm plate: N/D				
Microscopic observation of the revertants: N/D				
Category: Motile				

Strain: RF129

Growth Condition Time Temp.	Culture Appearance	Dominant Morphology	Other Morphology	Motility Description
21h 42 °C	slight turbid	short	-	Non-motile. No spinning and no swimming.
45h 42 °C	turbid	short	noodles, filaments	No swimming or spinning.
72h 42 °C	pale pink	short, elongated	noodles filaments	No spinning or swimming but some look slightly motile.
Colony appearance on swarm plate: Small swarms with revertant colonies growing from their edges.				
Colony appearance of the revertants on swarm plate: Small fuzzy colonies.				
Microscopic observation of the revertants: At first, they are mostly non-motile. But as they grow older they seem to become motile.				
Electron microscopy: Short cells changing to noodles and filaments with normal flagella.				
Category: Motile				

Strain: RF148

Growth Condition Time Temp.	Culture Appearance	Dominant Morphology	Other Morphology	Motility Description
24h 38 °C	slight turbid	short	elongated	Seem slightly motile.
45h 38 °C	turbid	elongated	short, filaments, noodles	Seem to be slightly motile.
72h 38 °C	pink	elongated	short	No swimming, no spinning. They seem to be non-motile.
Colony appearance on swarm plate: No swarms out of 1152 colonies. They are all sharp colonies.				
Colony appearance of the revertants on swarm plate: N/A				
Microscopic observation of the revertants: N/A				
Electron microscopy: Short and elongated cells with normal looking flagella.				
Category: Non motile				

Strain: RF151

Growth Condition Time Temp.	Culture Appearance	Dominant Morphology	Other Morphology	Motility Description
21h 42 °C	slight turbid	short, elongated	filaments	Non-motile, no spinning, no swimming. Some are slightly motile.
45h 42 °C	turbid	filaments, noodles	short, elongated	Non-motile. No spinning, no swimming. Some shorter ones seem to be jiggling.
72h 42 °C	pale pink	filaments, noodles	short, elongated	Non-motile. No swimming or spinning. They seem to jiggle where they are.
Colony appearance on swarm plate: Sharp to normal small swarms.				
Colony appearance of the revertants on swarm plate: N/D				
Microscopic observation of the revertants: N/D				
Category: Non-motile				

Strain: RF154

Growth Condition Time Temp.	Culture Appearance	Dominant Morphology	Other Morphology	Motility Description
24h 42 °C	slight turbid	short	elongated, filament, noodles	Most of the cells are non-motile but a few oscillate and spin very slowly.
45h 42 °C	turning pink	short	elongate, filaments, noodles	Most of the cells are non-motile; still some spin, swim or oscillate very slowly.
72h 42 °C	pink	short, elongated	noodles, filaments	Non-motile. A couple of noodles oscillate very slowly.
Colony appearance on swarm plate: Five swarms out of 1640 colonies that are sharp and non motile.				
Colony appearance of the revertants on swarm plate: N/A				
Microscopic observation of the revertants: N/A				
Category: Non-motile				

Strain: RF156

Growth Condition Time Temp.	Culture Appearance	Dominant Morphology	Other Morphology	Motility Description
21h 42 °C	slight turbid	elongated	short	Motile. Their motility is quite different than that of the wild type. They spin in one direction.
45h 42 °C	turbid	elongated	short, filaments	Spinning fast in one direction. The shorter, the faster.
72h 42 °C	pale pink	filaments, noodles	short, elongated	Shorter cells spin but many often seems to be non-motile.
Colony appearance on swarm plate: Medium size swarms.				
Colony appearance of the revertants on swarm plate: N/D				
Microscopic observation of the revertants: N/D				
Category: Non-motile to Motile				

Strain: RF160

Growth Condition Time Temp.	Culture Appearance	Dominant Morphology	Other Morphology	Motility Description
21h 42 °C	slight turbid	short	elongated	Non-motile.
45h 42 °C	turbid	elongated	short, noodles, filaments	Hard to tell if they are motile.
72h 42 °C	pale pink	elongated	short, noodles, filaments	Some seem to be very slightly motile.
Colony appearance on swarm plate: Three swarms out of 365 colonies.				
Colony appearance of the revertants on swarm plate: Pigmented swarms with fuzzy edges and dotted regions.				
Microscopic observation of the revertants: Short to filamentous cells that are very slightly motile.				
Category: Non-motile to Motile				

Strain: RF163

Growth Condition Time Temp.	Culture Appearance	Dominant Morphology	Other Morphology	Motility Description
21h 42 °C	slight turbid	elongated	short	Non motile, no spinning, no swimming. A few shorter ones seem to be very slightly motile.
45h 42 °C	very turbid	filaments, noodles	short, elongated	Non-motile. No spinning, no swimming. Some shorter cells and noodles seem to be slightly motile.
72h 42 °C	pale pink	filaments, noodles	short, elongated	Non motile. No swimming or spinning but it seems that some jiggle where they are.
Colony appearance on swarm plate: Fuzzy swarms with diameter of 2-4 mm with a dot in the centre.				
Colony appearance of the revertants on swarm plate: Small swarms, new swarms growing on the edge of the older one.				
Microscopic observation of the revertants: No swimming or spinning. Slightly motile if at all.				
Category: Non-motile				

Strain: RF169

Growth Condition Time Temp.	Culture Appearance	Dominant Morphology	Other Morphology	Motility Description
21h 42 °C	slight turbid	elongated	short	Non-motile, no spinning, no swimming. Some turn around.
45h 42 °C	turbid	filaments, noodles	short, elongated	Non-motile. No spinning, no swimming. Some shorter ones seem to be jiggling.
72h 42 °C	pale pink	filaments, noodles	short, elongated	Non-motile. No swimming or spinning. They seem to jiggle where they are.
Colony appearance on swarm plate: Sharp to normal large swarms.				
Colony appearance of the revertants on swarm plate: N/D				
Microscopic observation of the revertants: N/D				
Category: Non-motile				

Strain: RF171

Growth Condition Time Temp.	Culture Appearance	Dominant Morphology	Other Morphology	Motility Description
21h 42 °C	slight turbid	short	elongated	Motile. Cells are spinning and swimming.
45h 42 °C	turbid	short, elongated	noodles, filaments	Motile.
72h 42 °C	pale pink	short, elongated	noodles, filaments	Motile. Very motile. The longer the cells, the less motile they are.
Colony appearance on swarm plate: Large normal-looking colonies.				
Colony appearance of the revertants on swarm plate: N/D				
Microscopic observation of the revertants: N/D				
Category: Motile				

Strain: RF177

Growth Condition Time Temp.	Culture Appearance	Dominant Morphology	Other Morphology	Motility Description
21h 42 °C	slight turbid	short	-	Non-motile.
45h 42 °C	pale pink	short	elongated, noodles, filaments	No spinning or swimming but some cells seem to be motile.
72h 42 °C	pale pink	short	elongated, noodles, filaments	They seem to be motile.
Colony appearance on swarm plate: One swarm out of 560 colonies.				
Colony appearance of the revertants on swarm plate: N/D				
Microscopic observation of the revertants: N/D				
Category: Non-motile to Motile.				

Strain: RF178

Growth Condition Time Temp.	Culture Appearance	Dominant Morphology	Other Morphology	Motility Description
24h 38 °C	light turbid	short	elongated	Non-motile
48h 38 °C	turbid	short	elongated, filaments	Non-motile. Some short cells seem to be spinning.
72h 38 °C	pale pink	short	elongated filaments	Non-motile
Colony appearance on swarm plate: Three swarms out of 1200 colonies that are sharp and non-motile.				
Colony appearance of the revertants on swarm plate: N/D				
Microscopic observation of the revertants: N/D				
Category: Non-motile				

Strain: RF181

Growth Condition Time Tem.	Culture Appearance	Dominant Morphology	Other Morphology	Motility Description
21h 42 °C	slight turbid	short	-	Non-motile, no spinning, no swimming.
45h 42 °C	turbid	filaments, noodles	short, elongated	Non-motile. No spinning, no swimming. Some shorter cells seem to be slightly motile.
72h 42 °C	pink	filaments, noodles	short, elongated	No swimming or spinning. Some seem to be motile.
Colony appearance on swarm plate: Ten swarms out of 320 sharp colonies.				
Colony appearance of the revertants on swarm plate: N/D				
Microscopic observation of the revertants: N/D				
Category: Non motile				

Strain: RF182

Growth Condition Time Tem.	Culture Appearance	Dominant Morphology	Other Morphology	Motility Description
21h 42 °C	slight turbid	short	elongated, filaments	Motile. Short cells are spinning.
45h 42 °C	turbid	short, elongated	elongated, filaments	Motile. They are not very motile. Shorter cells spin and the longer the cells the slower they are.
72h 42 °C	pale pink	noodles, filaments	short, elongated	Motile. Short cells spin and longer ones oscillate.
Colony appearance on swarm plate: Medium swarms.				
Colony appearance of the revertants on swarm plate: N/D				
Microscopic observation of the revertants: N/D				
Category: Motile				

Strain: RF186

Growth Condition Time Temp.	Culture Appearance	Dominant Morphology	Other Morphology	Motility Description
21h 42 °C	slight turbid	short	elongated	Motile. Some cells swim straight. No cells spinning.
45h 42 °C	turbid	short	elongated. noodles, filaments	Motile. Some cells spin fast. Longer cells oscillate.
72h 42 °C	pale pink	short	elongated, noodles, filaments	Motile. Shorter cells spin fast and the longer cells swim slowly.
Colony appearance on swarm plate: Large pigmented normal swarms.				
Colony appearance of the revertants on swarm plate: N/D				
Microscopic observation of the revertants: N/D				
Category: Motile				

Strain: RF187

Growth Condition Time Temp.	Culture Appearance	Dominant Morphology	Other Morphology	Motility Description
21h 42 °C	slight turbid	short	elongated, noodles, filaments	Non-motile.
45h 42 °C	turbid	noodles, filaments	short, elongated	No spinning or swimming but some cells seem to be jiggling.
72h 42 °C	pale pink	noodles, filaments	short, elongated	They seem to be slightly motile. It is hard to decide whether they are motile or not.
Colony appearance on swarm plate: Small swarms.				
Colony appearance of the revertants on swarm plate: N/D				
Microscopic observation of the revertants: N/D				
Category: Non-motile to Motile ?				

Strain: RF188

Growth Condition Time Temp.	Culture Appearance	Dominant Morphology	Other Morphology	Motility Description
24h 38 °C	slight turbid	short, elongated	-	Non-motile. Some seem to be very slightly motile.
45h 38 °C	turbid	short	elongated, noodles, filaments	Some spin, they seem to be motile.
72h 38 °C	pale pink	short	elongated. noodles, filaments	Non-motile although there are some that seem to be very slightly motile.
Colony appearance on swarm plate: Two swarms out of 1100 colonies which are sharp and non motile.				
Colony appearance of the revertants on swarm plate: N/A				
Microscopic observation of the revertants: N/A				
Category: Non-motile				

Strain: RF189

Growth Condition Time Temp.	Culture Appearance	Dominant Morphology	Other Morphology	Motility Description
20h 38 °C	slight turbid	short	elongated	Motile.
48h 38 °C	turbid	elongated	short, noodles, filaments	Motile. The shorter the cells, the faster.
72h 38 °C	pale pink	elongated	short, noodles, filaments	Motile. Some cells spin.
Colony appearance on swarm plate: Non-motile. No swarms out of 1220 colonies.				
Colony appearance of the revertants on swarm plate: N/D				
Microscopic observation of the revertants: N/D				
Category: Motile ?				

Strain: RF190

Growth Condition Time Temp.	Culture Appearance	Dominant Morphology	Other Morphology	Motility Description
21h 42 °C	slight turbid	short, elongated	-	Motile. They are spinning and swimming.
45h 42 °C	turbid	short, elongated	noodles, filaments	Motile. Shorter cells spin and swim and the filaments oscillate.
72h 42 °C	pale pink	short, elongated	noodles, filaments	Motile. Short cells spin and longer ones oscillate.
Colony appearance on swarm plate: Medium swarms.				
Colony appearance of the revertants on swarm plate: N/D				
Microscopic observation of the revertants: N/D				
Category: Motile				

Strain: RF197

Growth Condition Time Temp.	Culture Appearance	Dominant Morphology	Other Morphology	Motility Description
21h 42 °C	slight turbid	elongated	short, filament, noodles	Non-motile, no spinning, no swimming.
45h 42 °C	turning pink	filaments, noodles	short, elongated	Non-motile. No spinning, no swimming. Some shorter ones seem to be jiggling.
72h 42 °C	pink	filaments, noodles	short, elongated	No swimming or spinning but it seems that some short ones jiggle where they are.
Colony appearance on swarm plate: Twenty-four swarms out of 402 colonies with fuzzy appearance.				
Colony appearance of the revertants on swarm plate: They all form swarms of small diameter of 2-5 mm.				
Microscopic observation of the revertants: Very slightly motile with no spinning or swimming.				
Electron microscopy: Short cells elongate with all intermediate shapes possessing normal flagella. Cells can mate.				
Category: Motile cell-cycle mutant.				

Strain: RF199

Growth Condition Time Temp.	Culture Appearance	Dominant Morphology	Other Morphology	Motility Description
21h 42 °C	slight turbid	short	elongated	Non-motile.
45h 42 °C	turbid	noodles, filaments	short, elongated	Very slightly motile.
72h 42 °C	turbid	noodles, filaments	short, elongated	Motile.
Colony appearance on swarm plate: Fourteen swarms out of 215 colonies.				
Colony appearance of the revertants on swarm plate: Colonies with fuzzy edges.				
Microscopic observation of the revertants: Very long filamentous cells that are very slightly motile.				
Category: Non-motile to motile				

Strain: RF201

Growth Condition Time Temp.	Culture Appearance	Dominant Morphology	Other Morphology	Motility Description
20h 38 °C	slight turbid	short	elongated, noodles	Spin and change sense of rotation. The elongated cells and noodles are very rare but as fast as the short ones.
48h 38 °C	turbid	short	elongated, noodles	The same.
72h 38 °C	turbid	short	elongated, noodles	The same.
Colony appearance on swarm plate: Very quickly form swarms.				
Colony appearance of the revertants on swarm plate: N/A				
Microscopic observation of the revertants: N/A				
Electron microscopy: Short and elongated cells with many flagellar filaments.				

Strain: RF202

Growth Condition Time Temp.	Culture Appearance	Dominant Morphology	Other Morphology	Motility Description
21h 42 °C	turbid	short, elongated	-	Short cells spin in both directions while longer ones oscillate and/or vibrate.
45h 42 °C	very turbid	short, elongated	-	Many spin, turn around, oscillate, swim forward, stop and change direction; Others jiggle in one spot.
72h 42 °C	pale pink	short	elongated	Spin very fast in both direction; Some swim, stop, spin and/or change direction and continue.
Colony appearance on swarm plate: Large swarms with rings in shorter time compared to the wild type.				
Colony appearance of the revertants on swarm plate: N/A				
Microscopic observation of the revertants: N/A				
Electron microscopy: Short cells with many long flagellar filaments.				

Strain: RF203

Growth Condition Time Temp.	Culture Appearance	Dominant Morphology	Other Morphology	Motility Description
21h 42 °C	slight turbid	short	elongated	Cells spin in both directions.
45h 42 °C	turning pink	short	elongated	Many spin in both directions very fast.
72h 42 °C	pale pink	short	elongated	some swim, some spin in both directions very fast.
Colony appearance on swarm plate: Needs less time compared to wild type to develop a large swarm.				
Colony appearance of the revertants on swarm plate: N/A				
Microscopic observation of the revertants: N/A				

Strain: RF204

Growth Condition Time Temp.	Culture Appearance	Dominant Morphology	Other Morphology	Motility Description
21h 42 °C	slight turbid	short	elongated	Cells spin in both directions.
45h 42 °C	turning pink	short	elongated	Many spin in both directions very fast.
72h 42 °C	pale pink	short	elongated	Some swim, some spin in both directions very fast.
Colony appearance on swarm plate: Needs less time compared to wild type to develop a large swarms.				
Colony appearance of the revertants on swarm plate: N/A				
Microscopic observation of the revertants: N/A				

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