Dottorato di Ricerca in Biologia Vegetale, XXI Ciclo (2005-2008)

Coordinatore: Prof. Aldo Musacchio

Tesi di Dottorato

Stressful and extreme environmental conditions: investigations on organisms from different domains

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INDEX

PREFACE AND AIM OF THE WORK

My PhD research project was focused on two different topics dealing with:

- A) The molecular bases of olive plant response to different stressful factors.
- B) The investigation of surface structure in *H.volcanii,* an archeon that lives in salt concentration close to saturation.

A) Olives are the most extensively cultivated fruit crop in the world. Cultivation area has tripled from 2.6 to 8.5 million hectares in the past 44 years. According to data from F.A.O., the ten largest producing countries are all located in the Mediterranean region and produce 95% of the world's olives. In particular Italy is the second world producer after Spain, with 1,140,685 hectares of cultivated area. The product of Olive cultivations, olive oil it is commonly used in cooking, cosmetics, pharmaceuticals, and soaps and as a fuel for traditional oil lamps. Italian and Spanish olive oils are the best known for their quality, especially the top-quality extra-virgin oils produced in Italy.

This study focuses on the stress conditions that have an impact on olive trees health, and therefore an impact on the cultivation of this

plant and the economy of olive oil production. To better understand when trees are under stress, and which particular condition affects the fitness of the plants, before the expression of a clear phenotype, we decided to begin our study at genomic level, using the approach of a candidate gene. A candidate gene is a gene whose effect is known to be relating to the biological systems and which might affect the traits of interest. We focused our attention on the dehydrin genes family. Dehydrins have been intensively studied during the past 10 years, trying to establish a correlation between their level of expression in numerous plants and different stress conditions. We decided to investigate the presence of the dehydrin genes in the Olive, for which no studies of those genes were reported.

We then decided to also work on dehydrin proteins expression, in order to test different stress conditions in a short amount of time through the use of a commercial anti-dehydrin antibody.

We subjected a pool of olive trees to different kinds of stress and we measured dehydrin expression, so to have a better understanding of how many dehydrins are expressed by the Olive trees during stress condition and if the expression of specific proteins is related to specific types of stress as opposed to being a generic response to stress of any kind.

B) Recent work has revealed that archaea have type IV pili-like structures. However the functions of these pili in archaea is not yet understood. In bacteria they are involved in crucial cellular processes including conjugation, autoaggregation, twitching motility and biofilm formation. The aim of this work is to determine whether the genetically and biochemically tractable archaeon *Haloferax volcanii has* predicted pili-like structures, and if so is able to form biofilms, autoaggregate and/or move by twitching motility. While archaeal biofilm formation has been reported in some archaea, the mechanism of formation of this complex structure is elusive. Moreover, there is no evidence of twitching motility in any archaeon.

Hence, upon identifying a large number of pilin-like proteins and pilus-biosynthesis genes, preliminary in vivo characterization of these subunits and electron microscopic analysis of *H. volcanii* surface structures, we focused our attention to the development of assays that would be used to determine whether archaeal type IV pili-like structures are also involved in these seminal cellular processes.

Using this haloarchaeal system, much has been learned about archaeal cellular processes such as transcription, protein transport and protein degradation. As the archaea share many characteristics with both the eukaryotes and bacteria, such analyses have already proven helpful in

elucidating the fundamental properties of these processes in all domains of life. Moreover, a better understanding of archaea, which often inhabit "extreme" environments, has enhanced the knowledge of the boundaries of biological processes of the biosphere, and has the potential to reveal information about the origin and evolution of the three domains of life.

THE OLIVE PLANT

Origin, taxonomy and distribution of *Olea europaea* L.

Olive is a member of the *Oleaceae*, the family that contains the genera *Fraxinus* (ash), *Forsythia* (golden bell), *Forestiera* (*F. neomexicana*, the California "wild- olive"), *Ligustrum* (privet),and *Syringa* (lilac) as well as *Olea* (olive). Commercial olives belong to the species *Olea europaea* L. There are about 20 species of *Olea* found in tropical and subtropical regions of the world, but only *O. europaea* L. produces edible fruit.

The origin of olive is lost in prewritten history.

The olive is one of the plants most cited in recorded literature. In Homer's Odyssey, Odysseus crawls beneath two shoots of olive that grow from a single stock (37). The Roman poet, Horace mentions it in reference to his own diet, which he describes as very simple: "As for me, olives, endives, and smooth mallows provide sustenance"(38). In Genesis, a dove released from the Ark by Noah, returned with an olive branch to show that the flood had receded. Thomas Jefferson wrote: "The olive tree is surely the richest gift of Heaven". The leafy branches of the olive tree, olive leaf as a symbol of abundance, glory and peace,

were used to crown the victors of friendly games and bloody war. As emblems of benediction and purification, they were also ritually offered to deities and powerful figures: some were even found in Tutankhamen's tomb.

Olive oil has long been considered sacred; it was used to anoint kings and athletes in ancient Greece. It was burnt in the sacred lamps of temples as well as being the "eternal flame" of the original Olympic Games. Victors in these games were crowned with its leaves. Today it is still used in many religious ceremonies.

From a systematic point of view *Olea europaea* belongs at the family of *Oleaceae* which includes 30 genera and 600 species (15).

The family *Oleaceae* was divided by Fiori and Paoletti in two tribes, *Jasmineae* and *Oleineae*, this latter including the genus *Olea* (Tourn.) On the basis of morphological features and geographical origins *Olea europaea* L., was classified in different taxa (28). The most extended taxa are:

- *O. europaea* ssp. *cuspidata* (Wall. ex G. Don), present in a big area from Southern Africa to Southern China;
- *O. africana* Mill. (Kleinhof ex Burm. F.), present from East to South Africa.
- *O. chrysophylla* Lam., which is extended between East Africa and Saudi Arabia.

In the temperate regions, the most common taxa are:

- *O. europaea* ssp. *europaea,* presents in the Mediterranean basin. *O. europaea* L. ssp. *sativa*, the cultivated olive, and *O. europaea* L. ssp. *sylvestris* (volg. Oleastro) belong to this group.
- *O. europaea* ssp. *Guanchica* typical of the Canary Island
- *O. europaea* ssp. *Laperrinei,* located in the mountains of central-southern area of the Sahara;
- *O. europaea* ssp. *Cerasiformis,* typical of the Madeira Island; it was reported like a subspecies already in 1993(29) and therafter confirmed like subspecies in 2000 (35) and 2001 (63).
- *O. maroccana* including the trees of the genus *Olea* present in South-west of Morocco whose taxonomy remains uncertain. (45)

Recently, the system of classification of *Olea,* based on morphological characters and phylogeographic data has been implemented with molecular studies that brought a big review in the *Olea* taxonomy. (35). In particular, a common origin between *O. Africana* and *O. europaea* ssp. *Europaea* was showed (5, 6).

Other studies also showed that the two North African subspecies, *O. laperrinei* and *O. cerasiformis*, resulted phylogenetically closer than previous reported in 1999 (3).

According to Besnard, (6), the genetic differences between taxa that are present in North Africa and those that are present in the

Mediterranean basin, could derive from the influence of climatic changes and human activities. Namely, these phenomena are already known to be part of the influence that in other species contributed at the selection of genetic difference (36).

The domesticated form *O. europaea* L most probably derives from the wild olives *Olea chrysophylla* Lam. and *O. europaea* L. var. *oleaster.* These wild types are known to have existed in the region of Syria about 6,000 years ago.(70). *Olea europaea* ssp*. sativa* is the only subspecie to have been cultivated since ancient times, representing one of more important plant for harvest of the Mediterranean basi

In Italy, olive production started in the VIII-VII century BC and gained economic importance with the input first of the Phoenicians and then of the Greek. Olive production developed during this time along the coastal and subcoastal areas of the eastern Mediterranean Sea, including souther European and Northern African countries. During Roman Empire the olive production advanced to the northern areas of Italy, Spain, France and Balkans (figure 1)(9).

Evolution through the millenniums of olive-tree production in the Near East and the Mediterranean basin

Today, Italian Olive production covers 1700 000 ha. The major part is located in southern Italy, where Puglia, Calabria and Sicily account more than 60% of Italian olive production. In term of olive oil production Italy is second in the world, and the Italian production is mainly represented by the extra-virgin and virgin olive oil.

Usually Olive prefers temperate climate zones, between 46° latitude north and 35° latitude south. It could be cultivated from sea level up to 600-700 meters of elevation. The plant is capable to resist at low temperature up to tra -6° and -8°C. Temperature below –10°C will cause severe damage to the entire plant. Taking into account the climate needs of different olive species, in Italy is possible to distinguish three different areas of olive production, where, due to the differences in climate, it is possible to record different olive

performance. The southern and insular area, the largest in terms of production, includes Sicily, Sardinia, Calabria, Basilicata and Puglia. This zone is considered the warm climate subarea for olive cultivation. In this area the favorable condition, plus the development of new technologies, allow to obtain high yields and high olive-oil quality, particularly in the production of extra-virgin oils.

Distribution of olive-cultivation areas in Italy with reference to climate

Internal zone of Sicily, Sardinia, Calabria, Basilicata and Puglia and the Italy's central-southern area (Lazio, Abruzzo, Campania and Molise), are considered as medium climate areas for olive cultivation. The environmental conditions of this area ensure optimal productivity. Moreover, yield is gained with supplemental irrigation, although in term of marketing the full value of the product is not reached yet, due to a lack of concentration of product.

The northern area of Italy, which includes the central-northern area of

Lazio, Marche, Liguria, Umbria, Tuscany, Emilia Romagna, Lombardy and Veneto is characterized by a lower oil production. This area is defined as cold subarea, due to the climate conditions, which are not suited to maximum productivity in terms of olive quantity and annual bearing (21).

Olea europaea ssp*. sativa* is an evergreen tree plant with long branches and leaves equipped with large stalk; the fruits are more or less developed according to the cultivar taken into account.

O. europaea L. ssp. *Sylvestris*, also known as oleaster, looks like a small shrub compared with the cultivated olive. He looks like a bush, with short branches and internodes; its ramifications are often grouped disorderly giving a sense of compactness. Both the leaves, which exhibit a short stalk, and the fruits, are smaller than those of *Olea europaea ssp sativa*.

Usually the Oleaster plants are present in area not influenced by cultivation. In some region, like Sardegna, they represent an important component of Mediterranean macchia ecosystem (1).

Morphology and vegetative development of *Olea europaea* L.

Olea europaea is a tree plant known for his longevity: in favorable climatic conditions it can became a millenary plant.

The germination of the seed is characterized by the emergence of embryonic root from the endocarp. Subsequently, the two cotyledons, free from endocarp, albumen and from the seed coat move toward the soil surface. At the end of the first year of life, the root system of seedling is formed by several side roots, which play an important role in the uptake of nutrients. The root system expands both vertically, reaching the depth of 1-1,5 meters and horizontally in the rhizosphere, occupying an area of up to two to three times the height of the plant (64).

Starting from third to fourth years of age, specific structures called ovules are formed on the stem, at the collar level. From these structures a new system of adventitious roots will develop and progressively substitute the primary one. In fact, in 7-10 years old plants the primary radical apparatus is known to necrotize (1).

The stem is characterized by a hypogeal part, called pedal, which usually grows under the ground level, and an epigeal part, called trunk, divided into branches. The distinction between the pedal and the trunk is not appreciable in young plants, becoming to be evident at 5-6 years of age. In adult plants, the pedal is clearly visible and takes the form of a big cone with an irregular basis.

In young plants the trunk looks slightly conical and has smooth, pale green-grayish, bark whose tone change in the different cultivars and

depending on the climatic zones. In adult plants the trunk loses its regularity and becomes contorted because of uneven radial growth, this makes difficult, almost impossible, the identification of well defined growth annuals ring.

The ramifications of stem are classified according to various criteria such as: age of the plant, connection between the ramifications with the trunk, direction of the ramifications, tendency to give wood or fruit production. Taking into account the age of the plant, as classification system, and adopting the nomenclature used for the plants to fruit, the ramifications, which form the crown of olive plants, are known as:

- old branches: big ramifications that are at least three years old.
- young branches: ramifications that are at least one year old
- sprouts: vegetative productions of the last year, which, very often, are not yet completely lignified. The old branches can be also distinguished in primary, when they originated directly from the trunk, and secondary when they originated from the primary branches.

Referring to the position occupied on the branch, the buds can be divided into top, auxiliary and adventitious. According to their biological activity, the buds are defined dormant, latent and ready. Lastly, based on the genetic information that they got, buds can be classified in vegetative buds, flower buds and mixed. However, the

fate of the buds is identifiable only in late spring, at the resumption of vegetative activity.

Olea europaea is a plant characterized by evergreen foliage. The formation of leaves occurs from spring to autumn and usually these persist on the plant just over a year, sometimes some remain even for two consecutive years. The age of the leaves is characterized by typical change of color. In fact, the adult leaves turn from green to dark green-yellow, to yellow during the final stages of age.

Olea produce on the same plant male and female flowers, which are grouped into inflorescence clusters. The inflorescences are green at the beginning, and then they turn into white and yellow.

The period of inflorescence development is distinctive for the various cultivars and depends on climatic factors. It is not synchronous on the same plant and usually begins in the branches exposed to the south. The pollen is round in shape and has a high vitality.

The pollination of *Olea europaea* is anemophilous, but despite the presence of hermaphrodites' flowers and the simultaneous maturing of sexual organs, self-imcompatibilty normally occurs. Thus most of the cultivars require cross-fertilization and the self-pollination is an exceptional occurrence. Self-imcompatibilty is related to the unability of pollen granules to germinate on stigma of female flower for the

presence of factors of incompatibility both in the genetic cells of the stylus as in that of pollen grains themselves (1).

.*Olea europaea* produces a fruit called drupe. The shape of drupe is fairly constant within the same cultivars while changes significantly in different cultivars. Some cultivars have drupe almost spherical, others oval, more or less straight or slightly arched.

Tipically, the drupe exhibits an epidermis, called epicarp, the pulp, mesocarp, and the woody endocarp. In the immature fruit the epicarp is green, but during full maturation it takes colors ranging from red to purple and black; less frequently it turns to white. The mesocarp consists of parenchymal cells, which increase in size as they proceed from the epicarp to the endocarp. Together with the parenchyma, in the mesocarp, can be found, sclereid cells, which can be isolated or organized into small groups. During maturation oil is accumulated in the mesocarp, together with phenolic compounds that contribute to improving the quality of the products, by virtue of their antioxidant properties. The endocarp is formed by lignified sclereids and encloses the seed. The seed consists of an outer layer, called seed coat, the endosperm and the embryo.

Olive's life cycle includes a youth (juvenile), maturity (adult) and finally a phase of aging (senescence). In the very youth phase, the trees exhibit morphological characters similar to those of wild forms,

but those disappear over the years. A peculiar feature of Juvenile phase is the unabilty of plants to flower. It is a very long phase that extends on average for 10-15 years. Consequently the iuvenile phase is one of the limiting factors in the programs for genetic improvement of olive (58). At the adult phase olive plants are able to undergo to both vegetative and reproductive development, which are progressively reduced in the aging phase.

Regarding the productivity of the olive plant, different stages can be distinguished:

- Phase of total unproductiveness, between 0 and 5-7 years;

-Increasing phase, that extends from 5-7anni until about 30 years and is characterized by a progressive increase in productivity;

- Stage of maturity, characterized by a remarkable productivity. It extends from about 50 years to more than 150 years;

- Beginning of senescence characterized by a progressive reduction in production capacity (64).

The division just described on the various phases of plant life cycle is quite indicative because the olive trees, even if senescent, can be reconstituted with rejuvenating operations, involving fertilization practices of the land.

In the annual productive cycle of adult olive plant the following stages can be distinguished:

- Resumption of vegetative development, which occurs in the spring. This phase is followed by flowering and subsequently by fruit development which occurs at 21 ° -22 ° C. Usually, less than 10% of flowers can develop into fruits);
- Phase of endocarp hardening, that occurs in summer, especially during the months of July and August;
- Autumn period, characterized by drupe maturation, which became characteristically green in color. During this phase the drupe increases its content in oil while reducing the water. Water and nutritional deficiencies occurring during this period, can significantly affect the harvest.
- Winter period during which the drupe turn from green to purple and finally to black, while the pulp becomes quite soft.

The aging of drupe happens gradually and is a very slow process, especially in the period when the solar irradiance decreases (64). With regard to its water needs, the olive tree shows a degree of efficiency in the use of water higher than that of many other species of tree fruit. In particular, the olive tree requires 312 grams of water to produce a gram of dry matter; citrus and plums will require respectively 30% and 75% more. Therefore, the olive tree is able to live in dry environments. However, in particular moments of its life cycle, adequate supplies of water are important for the growth and

productivity of olive plants. For example, during the development of drupe, up to the stone hardening, excessive lack of water causes a slowdown in growth, a fall or a small size of fruits and a low oil yield.

A BRIEF OVERVIEW OF PLANT RESPONSE TO STRESSFUL CONDITIONS

Stress and stress factors definition

Plants grow in several different environments and therefore are subject to a wide variety of stressful conditions that undermine their chances of survival and development. Stress conditions are defined as a significant deviation from optimal conditions for life. It causes physiological changes and responses at all levels of the functional organization. Initially reversible, those changes could become permanent with the continuing state of discomfort (43).

Plants possess a variety of protection mechanisms to delay or prevent the disorder of physical-chemical equilibrium between extra cellular and intracellular environment. In fact, the external factors, inducers of stress, never reach the protoplasm immediately or in their original

intensity. In condition of disorder, a series of resistance and protective mechanisms are also induced in the protoplasm to prevent or avoid the trouble (59).

Alternatively, the stress can be defined as a dynamic functional state, which includes destabilizing and destructive events that promote resistance countermeasure (59). In agreement with the dynamic concept of stress, it possible to identify a succession of phases that characterize this condition: the alarm stage, the phase of resistance and the stage of regeneration. The stage of alarm is the initial phase of the disorder and includes the structural (proteins, bio-membranes) and functional destabilization (biochemical processes, energy metabolism) of the cell that undermines the normal pursuit of viable activities. In the alarm phase the catabolic processes predominate over anabolic. If the intensity of stimulus remains unchanged, plant will establish the phase of cell refund that includes repair processes, as well as the synthesis of proteins or de novo synthesis of protective substances (43).

If the stress persist, then the situation will leads to the phase of resistance, but if the status of stress is too long or very intense, the plant can became susceptible to damage and diseases. However if the weakening is induced only temporary, original functional status can be restored during regeneration.

Stress also promotes the development of better-adapted genotypes. In populations exposed to prolonged stress the change from a reproductive strategy to one of survival, can be observed. It 'likely that any place of the earth's surface is a potential source of disorder and that moderates stress are thus an integral part of life, not a state of exception.

Stress factors can be divided into abiotic and biotic. Abiotic factors include: high or low radiation, extremes temperature (hot or cold), snow, ice, the low rainfall and drought. At the ground level, plants may be subjected to high concentrations of salts and minerals or mineral deficiency. Over-arid or alkaline soils are unfair and stressful for most plants. Among the abiotic factors are also: unstable soils, the movement of sand and finally the water that creates mechanical stress and deficit of oxygen, typical of flooded soils and bottoms of ponds and lakes.

Among the main biotic factors inducers of "stress" there are the microorganisms such as bacteria, fungi and viruses. Environments where the plants suffer grazing and poaching by animals belong to this category of stress. Typical biotic stresses take also place in area with dense phytomass where, for example, plants compete for the availability of light. Finally, the man is one of the factors responsible

for biotic stress, as producer of mechanical, physical and chemical pollution.

Within the several factors responsible for the stress, specific factors are those involved in well-defined target within the plant. In any case, the symptoms mentioned are highly specific and mechanisms of resistance induced involving all functional levels.(43)

Evolution of desiccation resistance

Keeping a correct function of the metabolic activity is a fundamental requirement for every organism, no matter in which kind of environment they live. For this reason the roles played by water are very important.

Desiccation tolerance in the vegetative tissues of plants has long been an area of intense interest for its relevance to agriculture and to the evolution of terrestrial plants. We can define desiccation tolerance as the ability to recover from the almost complete loss (80–90%) of protoplasmic water. This phenomenon is common in the reproductive structures of green plants such as pollen, spores and seeds. However, the ability to survive desiccation in the vegetative stage is a widespread but generally uncommon occurrence in the plant kingdom (7, 8).

The majority of vegetative desiccation-tolerant plants are found in the less complex clades that constitute the algae, lichens and bryophytes. However only in the gymnosperms there are no species that have desiccation tolerant vegetative tissues (51).

Recent synthetic phylogenetic analyses suggest that vegetative desiccation tolerance was primitively present in the bryophytes but was then lost in the evolution of tracheophytes.

The initial evolution of vegetative desiccation tolerance could represent a crucial step required for the colonization of the land by primitive plants from a fresh water origin(47). However, tolerance came at a cost, since metabolic rates in tolerant plants are low compared to those in desiccation-sensitive plants. On the other hand the loss of tolerance might have been favored the process that brought the vascular plants to become more complex.

So far, there are evidences that different independent evolution (or re-evolution) of desiccation tolerance occurred in *Selaginella*, in the ferns and least in eight independent angiosperms (2, 13, 51). These events are said to be independent since the natural rates of desiccation, re-hydration and responses to dehydration are different in each of these lineages (51).

The mechanism for achieving desiccation tolerance is very complex, but always involves two different key elements. The first response to

desiccative stress is an orderly and synchronous arrest of the plant metabolism to avoid accumulation of dangerous free radicals that could be generated by oxidation. For this reason desiccation tolerant plants stop photosynthesis while still retaining water during the desiccation process. On the other hand, during the period of desiccation, the cell have to preserve a good cellular organization in order to go back to normal metabolism rates after the re-hydration. Consequently, enzymes and membranes have to be protected from loss of functional configuration and the damage provoked by degrading non-metabolic reaction needs to be repaired. Under stressful conditions plants modify gene expression, increasing the expression of genes involved in defensive mechanism. This modulation of gene expression allows plants to survive in non-optimal conditions. For this reason one of the first process occurring during desiccation is the synthesis of new proteins involved in defensive mechanism. Several genes specifically expressed in this phase have been identified.

DEHYDRINS

Among the genes that are highly express during water stress conditions, there are those that transcribe for a set of hydrophilic proteins called late embryogenesis abundant (LEA) polypeptides (22-24). These proteins have long been implicated in cellular protection during both seed desiccation and water stress. LEA proteins of group II (14) also known as D_11 family of LEA proteins (19) are the most studied among drought induced water-soluble proteins. These proteins have been called "dehydrins" and have been observed in over 100 independent studies on drought stress, cold acclimation, salinity stress, embryo development and responses to ABA. (Close et al. 1989).

Dehydrins have a wide range of molecular masses, from 9 to 200 kD, (49) and are thermo stable proteins, able to maintain their integrity in aqueous solutions at up to 100°C. Generally, dehydrins are enriched with glycine and lysine residues, but they lack cysteine and tryptophan. The amino acid composition of these proteins is characterized by high content of charged and polar residues, and this determines their biochemical properties including thermo stability. This

may promote their specific protective functions under conditions of cell dehydration: dehydrins may prevent coagulation of macromolecules and maintain integrity of crucial cell structure.

Dehydrins are widely distributed in plants. Although some functions of dehydrins remain unknown, their induction in vegetative tissues during cell dehydration suggests their involvement in protective reactions. During water deficit, transcription of dehydrin genes is significantly higher in drought-tolerant than in drought-sensitive species. Correlation between drought tolerance of plants and dehydrin accumulation was found in sorghum plants like in sunflowers
(11, 68). In salt-tolerant lines of rice, the level of ABA-induced dehydrin expression was significantly higher than in sensitive plants (48). Moreover, a direct interrelationship between the level of dehydrins and cold tolerance has been observed (39, 55, 67, 69).

The involvement of dehydrins in the regulation of cell osmotic potential has been showed in various tissues of Arabidopsis and it is possibly related to their synthesis during water stress (reference).

Dehydrins are characterized by the presence of conserved domains in their structure. The most important domain common to all dehydrins is the K-segment, which is a highly conserved Lysine-rich motif consisting of 15 amino acid residues (EKKGIMDKIKEKLPG). However, some single amino acid substitutions and structural modifications may

occur within the segment. The number of K-segment repeats may vary from 1 to 12 (13, 60).

Many dehydrins also contain an S-segment that consists of serine residues; this site may be phosphorylated, so it could promote dehydrin interaction with specific signal peptides.

In most dehydrins it is present at their N-terminus, the Y-segment. The amino acid sequence of this domain shows similarity to portions of the nucleotide-binding site of chaperones of plants and bacteria, but its function is not yet clear.

Based on the presence of these highly conserved segments and their combination, dehydrins have been subdivided into five classes: YnSKn, YnKn, SKn, Kn and KnS (14). The precise function of each dehydrin type *in planta* has not been yet established. To get more insight in the potential roles played by DHNs in plants, their cryoprotective properties, ability to bind lipids and metals, and antioxidative activity have been thoroughly analyzed in *in vitro* studies (57). The main question arising from *in vitro* findings is whether each DHN structural type could possess a specific function and tissue distribution. Recent *in vitro* studies indicates that dehydrins belonging to different subclasses exhibit distinct functions (57).

YSK2-type dehydrins bind lipids *in vitro*: In pure form, DHNs seems to be intrinsically unstructured (40, 44), but they may form intrinsic (10)

structures when bound to target molecules (25). The K-segment present in all DHNs resembles a lipid-binding class A2 amphipathic α-helical segment found in proteins that have the ability to bind to acidic phospholipids and vesicles with small diameters. Since this capacity rises from the pronounced α-helicity, the two K-segments present in this class might be involved in membrane binding (10, 41).

KnS-type dehydrins have an *in vitro* radical-scavenging activity: Lipid peroxidation is a free radical-mediated degradative process that involves polyunsaturated fatty acids and results in the formation of lipid radicals. Some studies suggested that dehydrins belonging to this class facilitates plant cold acclimation by acting as radical-scavenging protein to protect membrane systems under low temperatures (30-32). KnS-type dehydrins also showed metal-binding activity. Thus, these proteins appear to functions also as radical-scavenger, reducing metal toxicity in plant cells under water-stressed conditions. Under conditions leading to the generation of hydroxyl radicals in plants this antioxidative activity may be a crucial function of KnS-type (42).

SKn- and Kn-type DHNs participate in plant acclimation to low temperature. It has been reported that some dehydrins display cryoprotective activity towards freezing-sensitive enzymes. For example the *Citrus unshiu* CuCOR19 dehydrin was shown to be more

effective in protecting catalase and lactate dehydrogenase against freezing inactivation than other compatible solutes (33).

MATERIALS AND METHODS

A) Western blot analysis

Plant materials

Two years old plants of *Olea europaea* L. cv Leccino were purchased from Santa Cruz Olive Tree Nursery (Watsonville, CA 95077). Plants were acclimated for 30 days at the Greenhouse of University of Pennsylvania, at 28 C and 50% humidity. Plants were watered.

Stress treatments

After the acclimation period the plants were divided into sub-groups that were exposed to different stress conditions. 3 plants were used as control, so they were grown at standard condition, to have always a visual control on the condition of the greenhouse.

For drought stress experiment 4 plants were subjected to water deprivation for 15 days. Proteins extraction was performed before the treatment start (control samples), and after 5, 10 and 15 days.

For salt stress experiments 3 plants were subjected to a final concentration of 200mM NaCl. Final salt concentrations were reached by adding 50mM NaCl per day to avoid salt shock. Also in this case

protein extraction was performed before the treatment and after 5, 10 ad 15 days.

For wounding stress experiments 3 plants were used. A small part of the leave was cut and the remaining part of the leave was collected and used for proteins extraction. Samples were collected before and at several time intervals (5, 30 minutes, 1 hour and 24 hours) after the application of the stress.

Sample preparation and proteins extraction

Proteins extraction was performed following the protocol published by Wang *et al.*, (65) with some modification. Olive leaves were ground in liquid N2 using a mortar. The powdered tissue was placed in microtubes and then resuspended in 1.0 mL cold acetone. Tubes were centrifuged at 10 000_*g* for 3 min (4∞C), after vortexing for 30 s. The pellet was washed again with cold acetone. After that, it was sequentially rinsed with cold 10% TCA in acetone 3–4 times or until the supernatant was colorless, then two times with cold aqueous 10% TCA, and finally with cold 80% acetone twice. Each time the pellet was re-suspended completely by vortexing and then centrifuged as above. The final pellet was dried at room temperature and used for protein extraction, or stored at -80∞C.

Phenol extraction of proteins (46), was performed in the presence of SDS (65). About 0.1 g of the dry powder of leaf tissue was re-suspended in 0.8 mL phenol (Tris-buffered, pH 8.0; Sigma St. Louis, MO, USA) and 0.8 mL dense SDS buffer (30% sucrose, 2% SDS, 0.1 M Tris-HCl, pH 8.0, 5% 2-mercaptoethanol) in a 2.0 mL microtube. The mixture was vortexed for 30 s and the phenol phase was separated by centrifugation at 10 000*g* for 3 min. The upper phenol phase was collected and 5 volumes of cold methanol plus 0.1 M ammonium acetate was added to the phenol phase. The mixture was stored at 20 ∞C for 30 min. Precipitated proteins were recovered at 10 000_*g* for 5 min, and then washed twice with cold ammonium acetate, in methanol, and cold 80% acetone, respectively. The final pellet was dried and dissolved in a buffer. Proteins concentration was quantified by the Bio-Rad ™ protein assay.

Electrophoresis and western blot

All protein samples were stored in 1 NuPAGE lithium dodecyl sulfate sample buffer and supplemented with 50 mM dithiothreitol. Samples were run on Bis-Tris NuPAGE gels (Invitrogen ®) under denaturing conditions using morpholinepropanesulfonic acid (MOPS). Proteins were transferred to polyvinylidene difluoride using the Bio-Rad

Transblot-SD semidry transfer cell at 15 V for 30 min. Three buffers were used in semidry transfer: anode I (300 mM Tris, 10% [vol/vol] methanol, pH 10.4), anode II (25 mM Tris, 10% [vol/vol] methanol, pH 10.4), and cathode (25 mM Tris, 40 mM glycine, and 10% [vol/vol] methanol, pH 9.4). Polyvinylidene difluoride membranes were probed with the primary antibodies Anti-Plant Dehydrin Polyclonal Antibody, (StressGen ™, San Diego, CA) (1:1,000), and the secondary antibodies anti-rabbit immunoglobulin (1:10,000). The K-segment synthetic peptide (TGEKKGIMDKIKEKLPGQH, *kindly provided by T.J. Close, Professor of genetics University of California, UC Riverside*) was used in the pre-incubation of the antiserum to confirm that all polypeptides observed contained the dehydrin consensus peptide (50).

B) *OeDEHYDRIN* CHARACTERIZATION

Plant materials

Ten years old plants of *Olea europaea* L. cv Carolea were used. These palnts derived by grafting from the same donor and belong to olive germoplasm collection of CRA (Centro di Ricerca per l'Olivicoltura) oin Rende (Calabria- Italy) and grown under field conditions

Total RNA extraction and reverse transcription

Total RNA was isolated from frozen tissues (100 mg) of leaves, was processed with the RNeasy Plant Mini kit® (Qiagen, Hilden, Germany) according to the manufacturer's instructions. In the elution step, RNA was resuspended in a volume of 50 µl of RNase-free water and incubated at 37°C for 30 min with DNase I in a final volume of 100 µl. DNase I was inactivated at 70°C for 15 min. RNA was precipitated and finally resuspended in 40 µl of RNase-free water. Quality and quantity of total isolated RNA were controlled with a NanoDrop® Spectrophotomer ND-1000. The total RNA (3-5 µg) from each sample was used, with the SuperScript III Reverse Trascriptase with the oligo dT(22), according to the manufacturer's instructions (Invitrogen, S.R.L., Life Technologies, Milan, Italy).

Isolation and sequence analysis of *Olea europaea* DHN cDNA

The cDNA was synthesised from leaf RNA (see sub-paragraph of RNA isolation and reverse trascription) and PCR-amplified by primers OeDHNFw (5'-GAAGGGGAAGGTTTCTGGAG-3') and OeDHNBw (5'-TCCTCAGTCTTGGGGTGGTA -3'). These were designed based on the class DHN on conserved amino acidic stretches EKKIGIMDKIKEKLPG (K-segment). The 276 base pairs (bp) fragment was cloned,

sequenced and confirmed to share high identity with DHN present in gene Bank.

The final PCR conditions were: 200 ng of cDNA, 1 mM of each primer, 0.5 mM dNTPs, Taq DNA polymerase (GoTaq Promega) 2.5 U, 1/10 of 10X Taq Buffer (Promega), 2.5 mM MgCl2, in a final volume of 50 µl. Cycling conditions included an initial cycle at 95°C for 5 min followed by 35 cycles at 95°C for 40s, either 55°C for 30-60s and 72°C for 1min, final extension at 72°C for 5 min. All PCR fragments were cloned into pGEM-T easy vector system (PROMEGA).

RESULTS

Characterization of a cDNA fragment of *OeDHN (Olea europaea* Dehydrin*)* belonging to the sub-class SK2

OeDHN partial cDNA was 276 bp long and encoded a deduced protein that consisted of 92 amino acids and contained the typical DHN domains (Fig. 1)

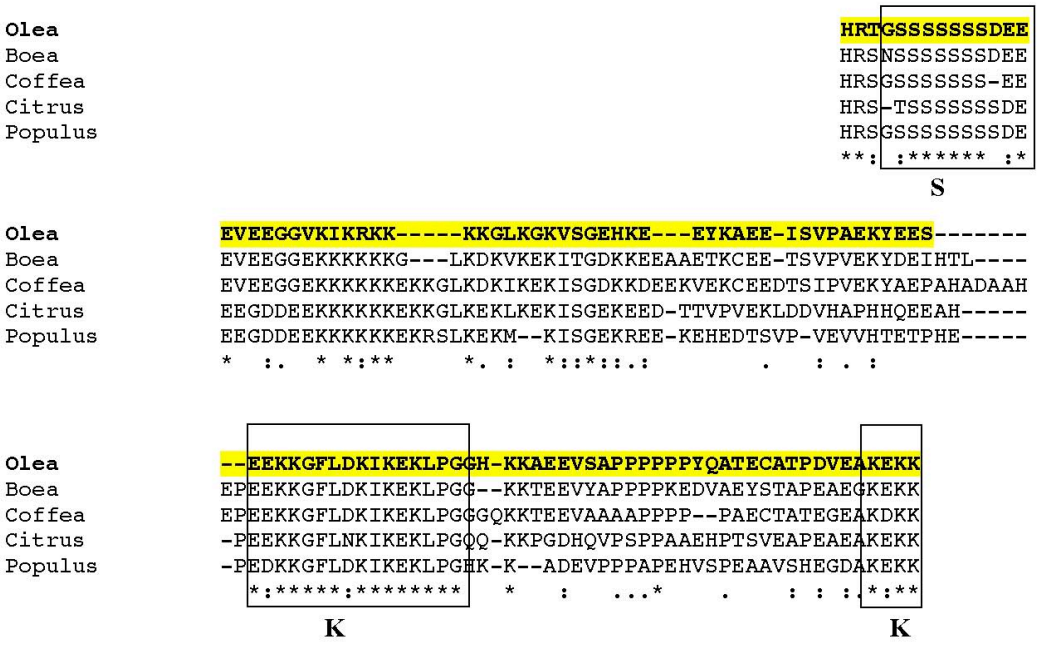

Fig:1. Alignment of *OeDHN* with the most closely related *DHNs* from *Boea crassifolia, Coffea canephora, Citrus and Populus x Canadensis*. *Olea europaea* is in bold. The conserved amino acid residues are shaded and the S- and K-segments are shown.

A GenBank Blastp search revealed that *OeDHN* had the highest sequence homology to the *Boea crassifolia* with 58% identity and 65% similarity. *OeDHN* also shared homology with *Coffea canephora DHN* (54% identity and 67% similarity), wild *Citrus (*50% identity and 63% similarity), *Populus x canadensis* (49% identity and 55% similarity). All these proteins which *OeDHN fragment* shared with the highest similarities belong to SK2 type *DHNs*. Thus, based on the one S-segments and two K-segments present in its amino acid sequence, *OeDHN* fragment might belong to SKn type *DHNs.* Work is in progress to fully characterize this gene

Salt stress condition induces expression of two dehydrin proteins

Western blot analysis, performed on protein extracted by leaves of cv. Leccino, using an anti-dehydrins antibody, show that at least two dehydrin proteins are expressed in the leaves of olive plants exposed to salt stress treatment (Fig.2A).

One protein, exhibiting a molecular weight around 42kDa, appears to be expressed, although at lower level, also in the plants before the beginning of treatment (control sample, 0 days), and then over-expressed under salt stress condition. Another small protein (18kDa) is expressed only under salt-stress conditions. During stress period the

expression levels of these two proteins increase over time and becomes particularly evident after 10 days of treatment.

To check the quality of the extraction procedure and so of the samples used, gel with the same amount of proteins was run and stained with Comassie Blue to (Fig.2B).

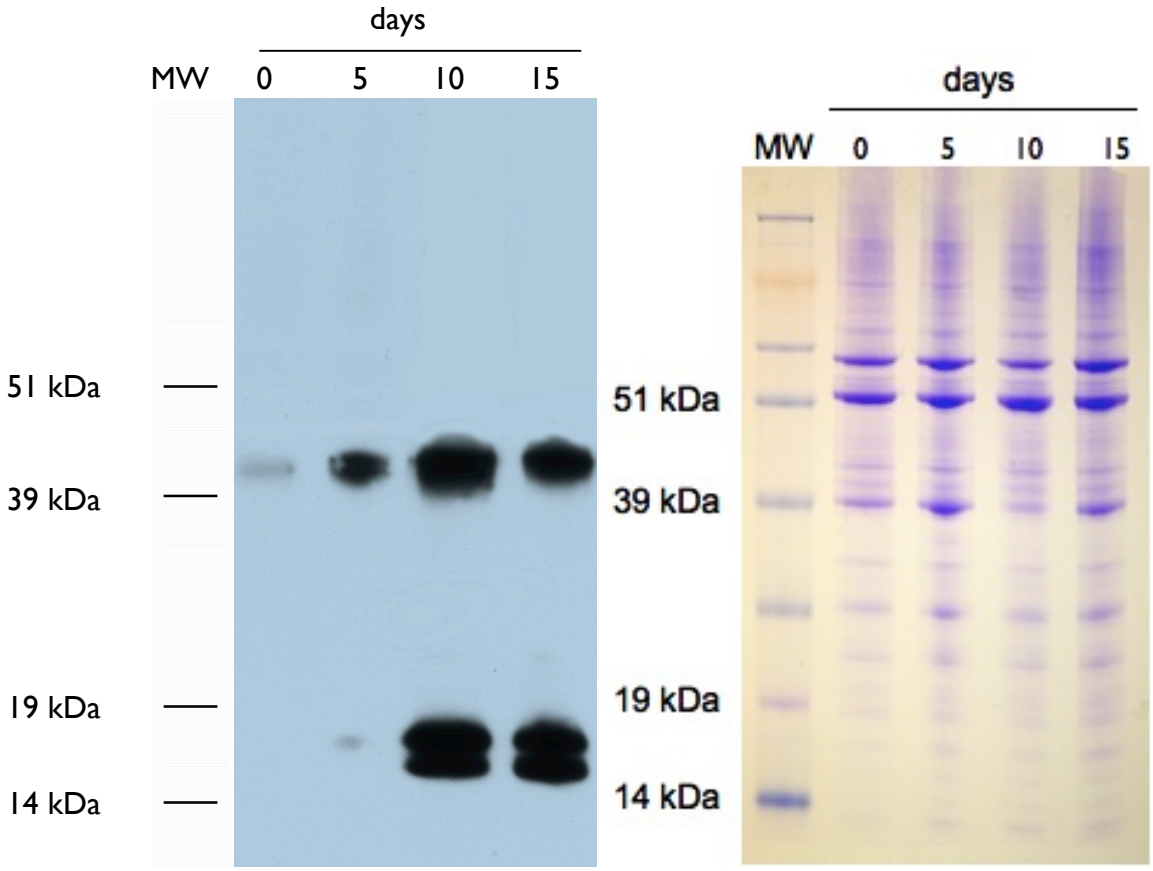

Fig.2A: Western blot analysis performed on proteins separated from: leaves collected before the treatment, 0 days, and leaves collected after 5, 10 and 15 days of salt stress (200mM NaCl). Protein were separated by SDS-Page and immunoblot was performed using anti-dehydrins antibody.
Fig.2B: Same amount of proteins were used for a Comassie Blue stained gel, to check the sample quality.

To confirm western result and to be sure that the bands visible on the western were due to the specific interaction between the the antibody and dehydrins, antibody was pre-incubated with a synthetic K-segment as a blocker . No bands were detected by using the pre-incubated immune serum (Fig.3A), thus confirming that all the observed proteins contain this dehydrin consensus peptide.

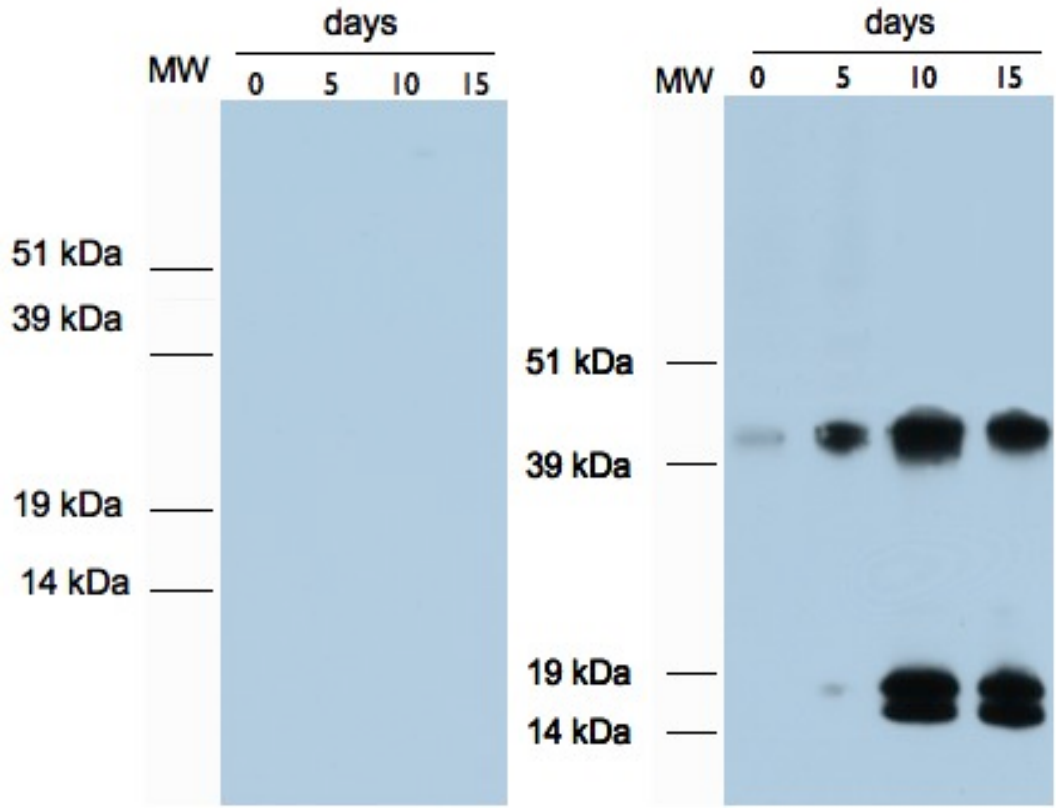

Fig.3A. SDS-PAGE was performed as describe in Fig.1, but this time the immunoblot was developed using the anty-dehydrins anti-body pre-incubated with the K-synthetic peptide. No band are recognize this time by the antybody.
Fig.3B. Western blot in Fig.1. is reported here again, to show the proteins recognized by the regular anti-dehydrin antibody.

Macroscopic effects of drought on olive leaves

One of the macroscopic effects induced on plants by drought stress is the visible symptom of leaf rolling. In olive plants this symptom have been observed in fully expanded leaves (Fig. 4A) after 7 days of dehydration (Fig.4B) and was massive after 15 days of treatment (Fig.4C).

Fig.4A: Visible symptoms induced in leaves of olive plants by drought treatment Control olive plant (Fig.4B) showing a healthy foliage. After 7 days of water deprivation leaf rolling can be appreciate (Fig.4C), and then becomes massive after 15 days ofreatment. Arrows indicate leaf rolling.

Drought stress induces dehydrins expression

Also in the case of water deprivation treatment, Western blot analysis, shows that at least two dehydrin proteins are expressed in olive leaves. In particular, one or two very close bands of about 42 kDa

were detected in the leaves of control plants not subjected to stress, but this (these) protein results over-expressed over time in the leaves of stressed plants. A new protein, not visible under normal condition, (18kDa) is detected in the leaves of water-deprived plants after 5 days of stress and its expression increases particularly after 15 days treatment (Fig.5A).

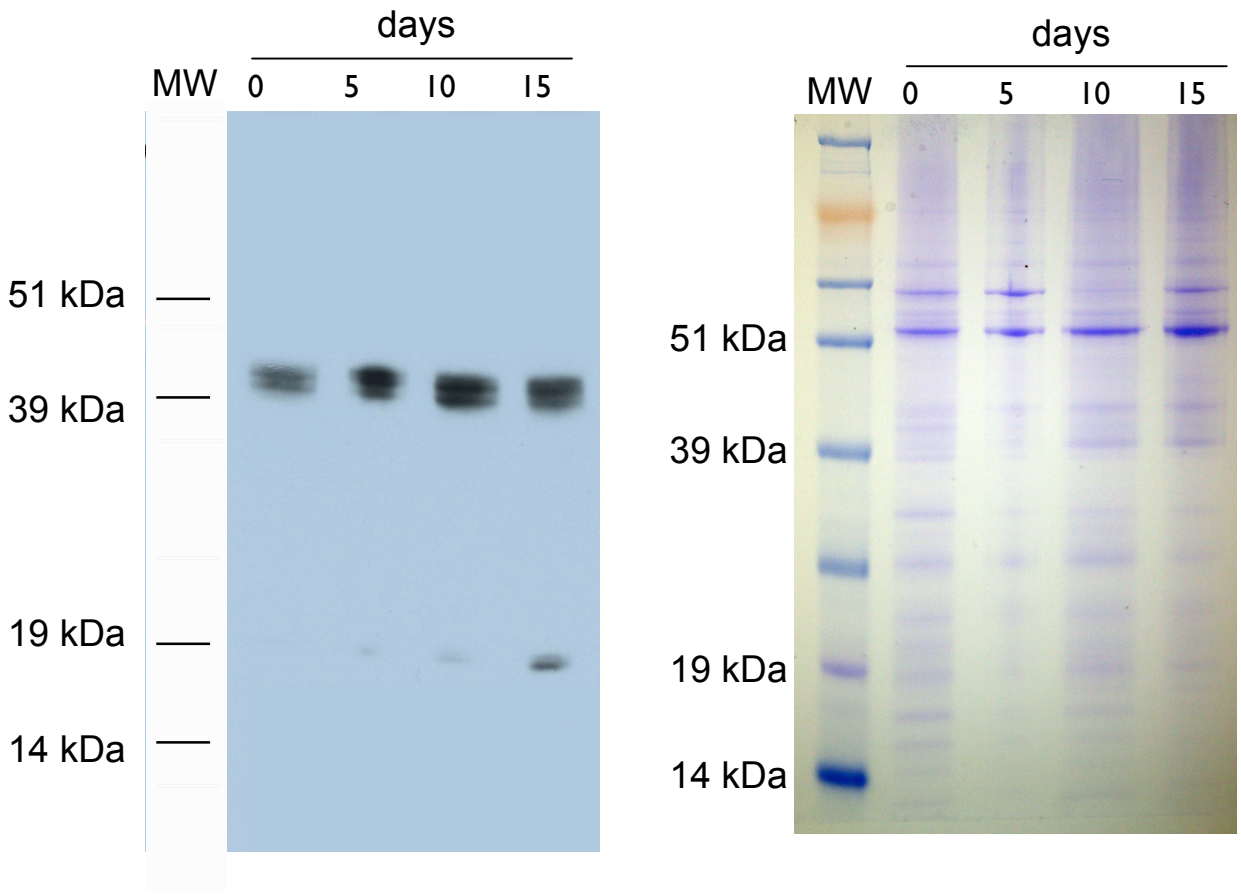

Fig. 5A: Olive leaves were collected before the treatment, and after 5, 10 and 15 days of water deprivation. Western blot was developed with the anti-dehydrins antibody.
Fig. 5B: Gels contain the same amount of protein in the western, was stained with Comassie blue to check the quality of the samples extraction.

Dehydrin expression after wounding

Plants are subject to various types of physical damage, derived from their sessile nature. Damage caused by insect feeding, wind, hail and other environmental stresses is commonly referred to as wounding(12) (53). Wounding stress was applied to olive's leaves and samples were collected as described above and used for western blot analysis.

Fig: 6A-B. Wounding treatment: Tips of the leaves were cut out, and the remainder of the leaves was collected at different interval of time. Arrows indicate zones where wounding was applied.

The obtained results show that wounding stress induces only one dehydrins (Fig. 7A). This protein of about 42 Kd, which as previously described, is constitutively expressed under normal condition (Fig.2),

is over expressed in wounded leaves and its expression peaks at 24 hours after wounding.

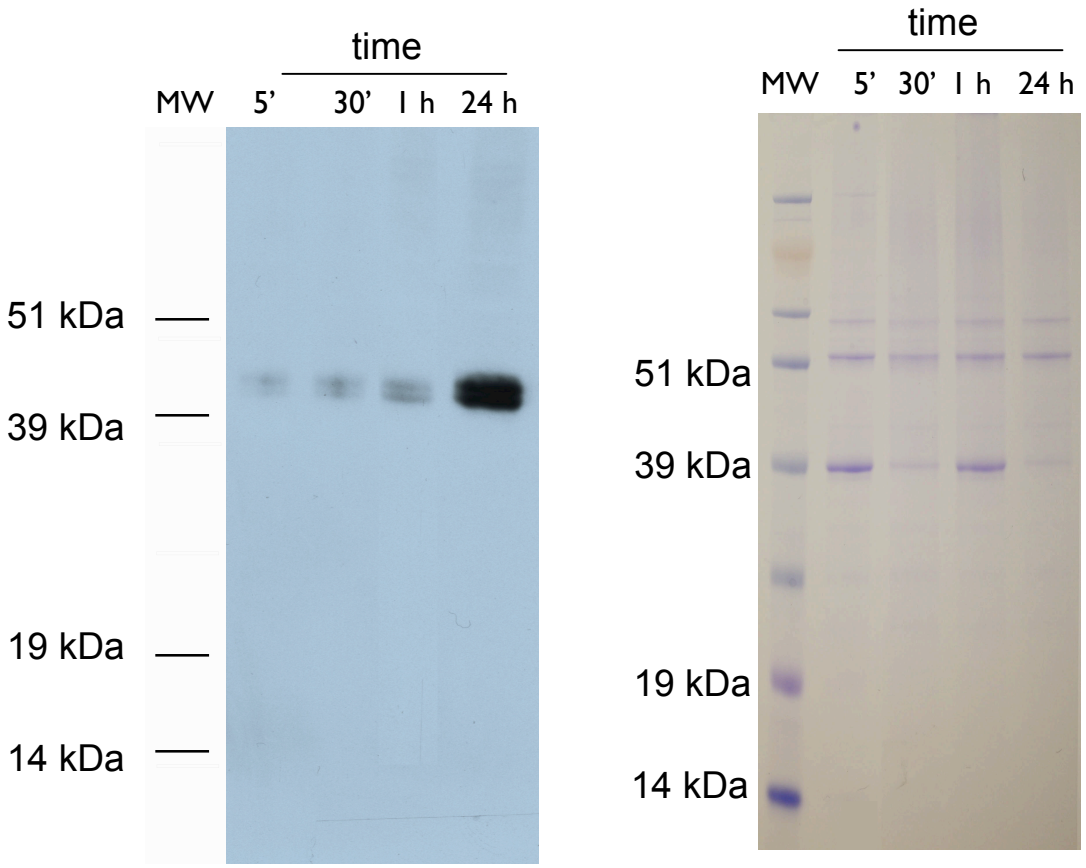

Fig: 7A: Western blot of leaves sample under wounding condition. Samples were collected after 5, 30 minutes, 1 hour and 24 hours. No difference were found between control and 5 minute of stressed samples.
Fig: 7B. Comassie Blu gel.

DISCUSSION AND CONCLUSIONS

The accumulation of dehydrin proteins and their transcripts are induced in plants tissues by different stresses (57). In this work we analyzed the expression of dehydrin proteins in *Olea europaea*'s leaves trough a Western blot analysis. Plants were subjected to different stresses, other than drought, which are known to induce dehydrin expression in different species, such as salt stress and wounding (27, 54). With our analysis we have established that at least two dehydrin proteins are expressed in *Olea europaea* leaves.

One dehydrin protein of about 42kDa was detected in leaf tissues in normal conditions, so it appeared to be expressed constitutively, according with other studies in different plants (17, 56). Expression levels of this protein increased when the olive plants were subjected to stress conditions. Indeed, its expression was significantly enhanced after 5 days of both salt and drought stress.

During these treatments a 18kDa dehydrin was also expressed in the leaves, while it was undetected in the leaves of plants grown under normal conditions. More precisely, this protein was detected after 5 days of stress, and its expression level increased significantly after 10 and 15 days days of salt stress treatment and water deprivation,

respectively.

In order to investigate whether the expression of 18kDa dehydrin was stress specific, we submitted the olive plants to a stressful condition, which didn't involve drought and osmotic regulation. In particular, we decided to apply a wounding stress, known to also induce dehydrin expression (12, 53). Wounding is a common damage that occurs in plants as a result of abiotic stressful factors, such as wind, rain, hail as well as of biotic factors, especially insect feeding. In this case we focused most on the abiotic effect of the wounding, since no pathogens were involved in this treatment but only mechanical damage.

Since this treatment was applied directly on the leaves, the response of the plant was supposed to be much faster, compared to that induced by drought and salt treatment. In this case, indeed, we could see the over expression of the 42kDa protein after 24 hours from the damage.

Interestingly the dehydrins of 18kDa, that was expressed under drought and salt stress, was not detected in wounded leaves, thus supporting the notion that dehydrins specifically respond to the different types of stress (57).

Our results also confirm that dehydrins proteins represent potential markers to identify early state of stress in plant. Therefore, future development on this aspect will aid the effort of plant breeding to

improve different kind of tolerance. Likely, *Olea europaea* can express more dehydrins than the ones seen in this work. Work is in progress to test dehydrin expression under other stress conditions such as low temperature, wounding followed by jasmonate treatment, or pathogens attack. Additionally, we planned to not only follow the expression of dehydrins in leaves but also in different parts of the plant, such as fruit (52) and roots.

Dehydrins have been referred to as "extremely hydrophilic" LEA and COR proteins (61), due to the presence of hydrophilic domain in their structure, but still little is known about their function. It is very interesting to note that these proteins seem to have no defined three-dimensional structure in their native state. They belong to the category of intrinsically unstructured proteins (20, 66), and become structured only in association with ligands of various types, including lipids, tubulin, and other proteins (62). There are numerous additional examples of proteins that appear to be "natively unfolded" in pure form but are structured in association with ligands of various types, including lipids, tubulin, and other proteins (for example, see table I of Weinreb et al., (66). Perhaps, by exploring hydrophobic interactions between dehydrins and their ligands the physiological roles of dehydrins could become better understood.

In our work we also began to investigate the presence of the

DHNs genes in the Olive genome, for which no studies of those genes were reported. At the moment, a cDNA- fragment of *OeDHN* gene, belonging to SKn type *DHNs* (52), has been characterized, thus proving that *a*t least one *DHN* gene is present in the olive tree . Work is in progress to fully characterize this gene as well as other members of *DHNs* family. Currentely, the characterisation of olive germoplasm is still far from being complete (4, 18); and only few genes related to physiological traits have been investigated (16, 26, 34). Hence, a wide characterization of genes related to stress response could provide knowledge and tools for breeding and biotechnological approaches.

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ARCHAEA

BACKGROUND

Organisms from the domain Archaea are well known for their ability to thrive under extreme conditions, such as high or low temperature, saturating salt concentrations, high pressure and high or low pH values (2).

However, relatively recent molecular studies have shown that Archaea, which are essential to ecological processes such as the carbon and nitrogen cycles, are ubiquitous and can be found in environments ranging from garden soil and coral reefs to the human colon and oral cavities (10, 17, 21). Today a good part of newly identified organisms belong to this domain of life.

The Domain Archaea wasn't recognized as a major domain of life until quite recently. In 1970, a system of Five Kingdoms was the accepted the model by which all living organisms could be classified. At a more fundamental level, a distinction was made between the prokaryotic bacteria and the four eukaryotic kingdoms (plants, animals, fungi, & protists).

Archaea were classified as a separate group of prokaryotes only in 1977 by Carl Woese and George E. Fox based on the sequences of a

ribosomal RNA gene(68). They named the two groups Archaebacteria and Eubacteria. But then Woese argued that Archaebacteria is a fundamentally different group of living organism.

To emphasize this difference, these two domains were later renamed Archaea and Bacteria (69).

Unfortunately, although archaea play profound roles in essential environmental processes and accumulating evidence indicates that they can have significant impact on human health, few studies on the molecular mechanisms underlying most archaeal cellular processes have been completed. For example, while it is known that archaea inhabit biofilms, complex mucilaginous structures that provide resistance to a variety of environmental stresses in ecosystems ranging from dental plaque to hydrothermal vents, nothing is known about the cellular processes used by Archaea to establish or inhabit biofilms (18, 27, 30, 54, 55, 66).

ORIGIN AND EVOLUTION

Probable fossils of archaeal cells have been dated to almost 3.5 billion years ago (59), and the remains of lipids that may be either archaeal or eukaryotic have been detected in shales dating back as far as 2.7

billion years (8). Due to the fact that most prokaryotes do not have distinctive morphologies, fossil shapes cannot be used to identify them as archaea or bacteria. Instead, chemical fossils, in the form of the unique lipids found in archaea (see below), are more informative because such compounds do not occur in other groups of organisms (11). Such lipids have now been detected in rocks dating back to the Precambrian. The oldest known traces of these lipids, found in west Greenland, include sediments formed 3.8 billion years ago and are the oldest on Earth (22). The origin of Archaea appears very old and the archaeal lineage may be the most ancient that exists on earth(67). Hence the word Archaea, that derives from the Ancient Greek ἀρχαῖα, which means "ancient things".

The relationship between archaea and eukaryotes remains an important problem. Aside from the difference in cell structure, many genetic trees group the two together. One possibility, which is prevalent right now, is that the ancestor of the eukaryotes diverged early from the Archaea (70). Moreover, it has been suggested that eukaryotes arose through fusion of an archaean and a bacterium; this accounts for various genetic similarities but runs into difficulties explaining cell structure (39). It has also been suggested that due to lateral gene transfer, a tree may not be the best representation of the genetic relationships of all organisms (48). The question about the

correlation between Archaea, Bacteria and Eukaryote is still an open debate.

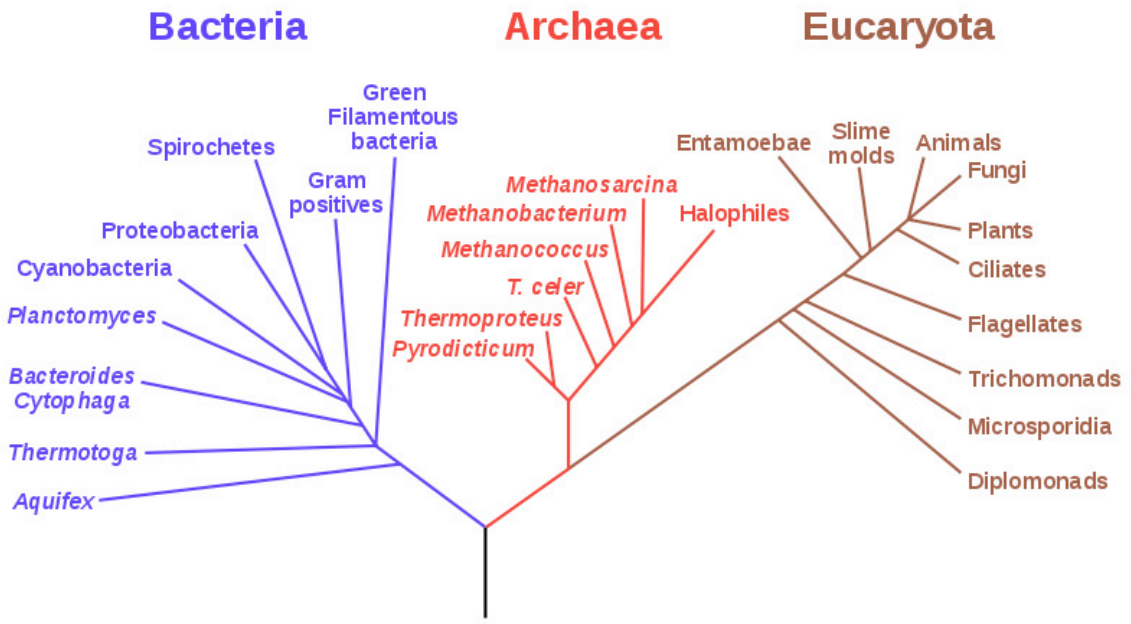

Figure 1: *A phylogenetic tree of living organisms, based on 16s rRNA data and proposed by Carl Woese, showing the separation of Bacteria, Archaea, and Eukaryote*

DIFFERENCE WITH THE OTHERS DOMAINS

Cell membranes

Molecules in archaeal membranes differ strongly from those in other forms of life (35). In all organisms cell membranes are made of molecules known as phospholipids.

The phospholipids in the membranes of Archaea are unusual. Bacteria and eukaryotes have membranes composed mainly of glycerol-ester lipids, while Archaea have membranes composed of glycerol-ether lipids(15). Ether bonds are chemically stronger than ester bonds, which might contribute to the ability of some archaea to survive at extremes of temperature and in very acidic or alkaline environments. Bacteria and eukaryotes do contain some ether lipids, but in contrast to archaea these lipids are not a major part of their membranes.

Another difference is the stereochemistry of the glycerol group. This molecule exist in two conformations called enantiomers, (right and left) and archaea use the L-glycerol, which require the existence of different enzymes for the synthesis of archaea phospholipids. The structure of the lipid tails in archaea is also different. In contrast to the fatty acids found in bacterial and eukaryal organisms' membranes, archaeal lipids have long chains with multiple side-branches. Only the Archaea use isoprenoids, in the place of fatty acid, to make phospholipids. Another interesting property of the side branches is their ability to form carbon rings. These branched chains may help membranes to stay tight at high temperatures(36). Finally, some thermophilic archaea replaced the phospholipid bilayer with a single monolayer. Those archaea have a single phospholipid molecule with

two polar heads; this may make their membranes more rigid and better able to resist high temperatures (24).

Metabolism

Organisms belonging to the archeal domain use diverse sources of energy and are capable of different chemical reactions in their metabolism. Archaea of the groups of nitrifiers, methanogens and anaerobic methane oxidisers for example, use inorganic compounds such as sulfur and ammonia as source of energy. Organisms belonging to this group are called lithotrophs(65). Other groups of archaea are phototrophs, using light to generate energy(58). One groups of Archaea, the methanogens, are able to produce methane gas. This anaerobic process of CO_2 fixation for methane production, involves different enzymes and co-enzymes that are unique to these archaea. Since this form of metabolism evolved early on the earth, it could be possible that the first free-living organism was a methanogen (34). Next to methanogen, other autotrophic archaea, (organism that use the CO_2 present in the atmosphere as a carbon source) have been identified. To be able to fix carbon, archaea use a highly-modified form of the Calvin cycle (45), or a recently-discovered metabolic pathway called the 3-hydroxypropionate/4-hydroxybutyrate cycle (6). The

energy sources used by archaea to fix carbon are extremely diverse, and range from the oxidation of ammonia to the oxidation of hydrogen sulfide or elemental sulfur (21, 38, 58).

The fact that many basic metabolic pathways are shared between all forms of life is probably an indication of the early evolution of these metabolic ways and their high level of efficiency (56).

HALOFERAX VOLCANII

Haloferax volcanii is a species of halophile which exists in extreme saline environments (46). This organism has been isolated from high-saline environments, such as the Dead Sea, the Great Salt Lake, and oceanic environments with high NaCl concentrations. *H. volcanii* is a moderate halophile that grows on simple defined media, is readily transformable and has a stable genome. This, in combination with its biochemical and genetic tractability, has made *H. volcanii* a unique model organism, not only for the study of halophilicity, but also for archaeal biology in general. The sequence of its genome, which is approximately 4000 open reading frames (ORFs), is completed and microarray data using various conditions are accumulating. *H. volcanii* can grow in a wide range of salt concentrations (1.5 – 3M) allowing

analysis of adaptation to high and relatively low salt concentration. Moreover, while even at the lowest feasible concentration of 1.5M NaCl certain protein purification schemes (*i.e.* ion exchange chromatography), are not usable, other purification methods have successfully been employed. For the particular project discussed here, it is noteworthy that *H. volcanii* is the only haloarchaeon in which, next to autoaggregation and surface adhesion, DNA transfer has been demonstrated (44, 57).

SURFACE STRUCTURES

Bacterial Type IV pili

Non-flagellar surface-structures were originally identified in gram-negative bacteria in the early 1950s by scanning electron microscopy (29). Extensive analysis of these pili (also called fimbriae) over the past decades revealed several distinct types. One of the best characterized of these are the type IV pili, which were first identified as polymers that traverse the outer membrane of gram-negative bacteria contributing to survival and pathogenesis. In fact it was found that these filaments, which are able to retract and extend, exhibit amazing multifunctionality, with roles in adhesion,

autoaggregation, surface (twitching) motility, biofilm formation, DNA uptake and bacteriophage attachment (7, 33, 42, 50).

In bacteria, type IV pili are comprised of distinct major and minor pilin subunits that are transported across the cytoplasmic membrane via the universally conserved Sec pathway. The N-terminal Sec signal peptide of type IV pili subunits (a class III signal peptide) targets these subunits for membrane translocation. The class III signal peptide consists of an N-terminal positively charged region followed by a highly hydrophobic region, with the two regions separated by a peptidase processing site (Fig: 2). Upon recognition of this signal peptide by the signal recognition particle (SRP), the pilin subunit is targeted to the Sec pore (20). Since these proteins are transported across the membrane in an SRP-dependent manner, it can be inferred that they are transported across the membrane in a co-translational fashion like many other Sec substrates. In contrast to the processing of class I and class II Sec signal peptides, once translocation of the immature pilin subunit occurs its class III signal peptides is processed *amino-terminal* to the hydrophobic region (Fig. 2). Retaining the hydrophobic domain as part of the mature protein is crucial as interaction of these domains with each other provides a molecular scaffold for the helical assembly of the subunits into the pilus fiber (14). Consistent with the essential role of the hydrophobic domain in

the assembly of the pilus, the amino acid sequence of this region exhibits a high degree of conservation(62).

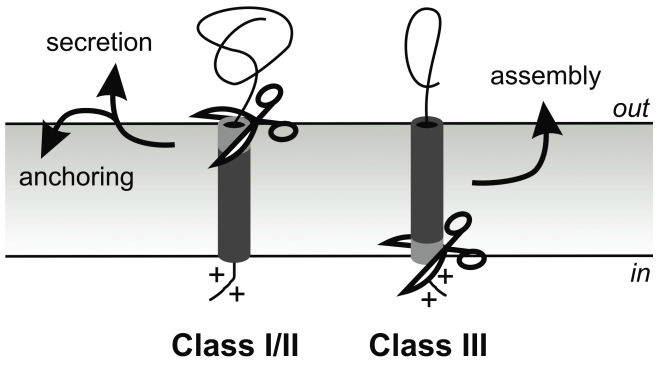

Figure 2: N-terminal signal peptide structures. *Structures of class I (secretory) or class II (lipoprotein) signal peptides, and class III (type IV pilin-like) signal peptide. Signal peptide cleavage by signal peptidase I and II, and prepilin peptidase (SPIII), respectively, is symbolized by scissors; dark grey, hydrophobic region, light grey, cleavage region; +, positive charges.* From Szabo *et al.(62).*

Type IV pili in bacteria can be several μm long and 5 -7 nm in diameter. These fibers are composed primarily of a single small protein subunit (~140-200 amino acids in length) that is assembled into a helical conformation in the periplasm of gram-negative and on the cell surface of gram-positive bacteria. In gram-negative bacteria, the pili protrude across the outer membrane through a proteinaceous pore (secretin). All type IV pili have a conserved structure, where the highly hydrophobic domain forms the core of the pilus fiber, and the outside of the fiber is formed by a highly organized scaffold of ß-sheets packed flat against the core with an outermost layer composed of an exposed hypervariable region and an extended C-terminal tail. While

the overall conservation of the structure allows the pilin subunits to assemble into a strong yet flexible filament, the hypervariable regions provide different functions of these surface structures within and among species (25, 41). In fact, the C-terminal variability may reflect selection pressures by pili-specific bacteriophages, adaptation to different environments, receptors, or simply evolutionary drift.

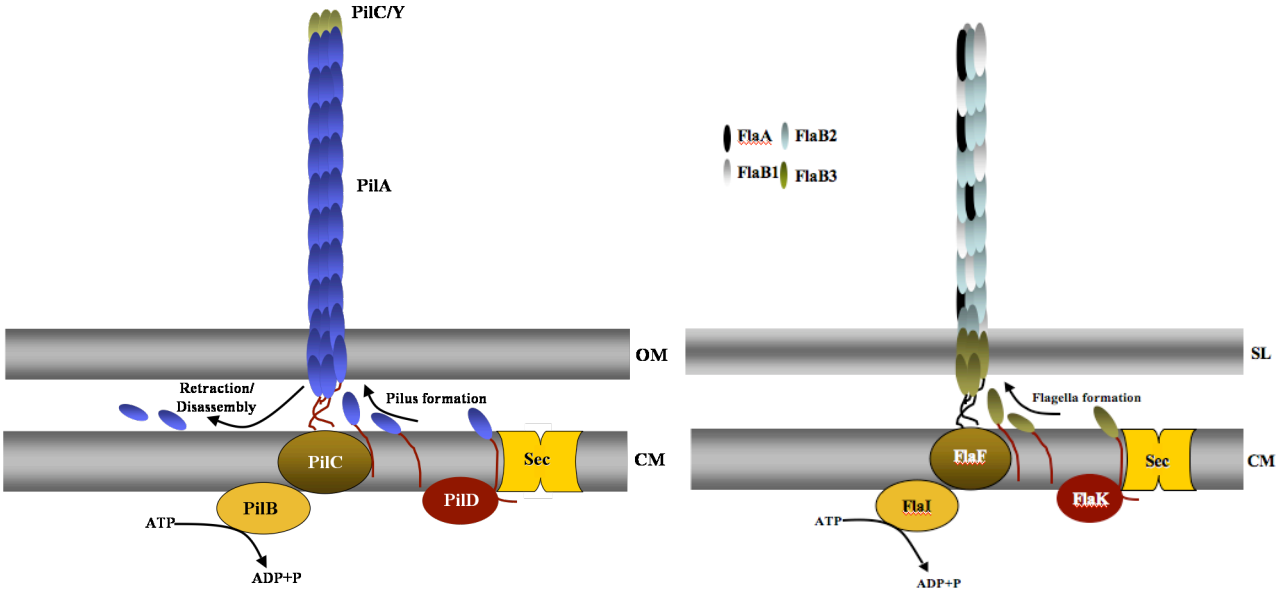

Figure 3A. Type IV pilus assembly and structure: *Type IV pili are composed primarily of a single small protein subunit (PilA) that is arranged in a helical conformation. Pilins contain class III signal peptides, which are secreted via the Sec pathway. The prepilin peptidase (PilD), cleaves the signal peptide N-terminal to a highly hydrophobic domain that subsequently forms the core of the pilus fiber on which a unique set of pilin structural motifs are anchored. In addition to the major pilin, several minor pilin-like proteins are important for the assembly and function (e.g. adhesion, PilC/Y) of the pilus. In addition to PilD, two conserved protein families are crucial for pilus biosynthesis: a VirB11-like ATPase (PilB), which provides energy for the assembly and disassembly of the pilus, and a multispanning membrane protein (PilC), which has been suggested to serve as an assembly platform for the pilus. CM:cytoplasmic membrane; OM: outer membrane.*
Figure 3B. Schematic representation of predicted composition and assembly of archaeal flagella, *(modified from Bardy et al. (5)). Archaeal flagellins possess class III signal peptides that are cleaved by a prepilin-peptidase homolog (FlaK) before the incorporation of the protein into the flagellar filament. Moreover, like type IV pili,*

the filament is generally composed of multiple flagellins that are present in drastically different stoichiometries. Finally, a PilB ATPase homolog, FlaI, and a multispanning membrane protein FlaF, which shows homology to PilC and might in a similar way serve as an assembly platform for the flagellum, are required for flagella biosynthesis. CM:cytoplasmic membrane; SL: S-layer.

In addition to the major pilin, several minor pilin-like proteins, containing the class III signal peptide are important for the assembly and function of the pili. For example, some subunits may provide the base or anchor for the pilus fiber, while others might be incorporated at the tip, where they might play a role in initiation or stabilization of pilus assembly. Alternatively, they may be involved in the prevention of pilus retraction, controlling pilus lengths or in pilus-specific functions such as adherence, competence or motility. In addition to the prepilin peptidase involved in the processing of the class III signal peptide, two conserved protein families are crucial for pilus biosynthesis: an ATPase (PilB) that provides energy for the assembly and disassembly of the pilus, and a multispanning membrane protein (PilC) that has been suggested to serve as an assembly platform for the pilus. Pili retraction is apparently stimulated by the activity of the ATPase PilT, a molecular antagonist to PilB. The mechanism by which hydrolysis of ATP is coupled to pilus assembly and disassembly is currently unknown.

Functions of type IV-pili: As noted above, type IV pili can reversibly assemble. Bacteria have taken advantage of the extension and

retraction capability of pili to accomplish a diverse set of functions. For example, once the distal end of a pilus attaches to a surface, retraction of the pilus provides a moving force for the bacterium that is known as twitching motility. This type of flagella-independent motility has been observed in a wide range of bacteria and is important for host colonization and complex colonial behavior. For example, it is required in the initial stages of biofilm formation (i.e., the formation of microcolonies) (see below) (7, 42). Bacteria also use these dynamic structures to form an initial association with host cells, that brings the bacterium into greater proximity of the host-cell surface and thus facilitate a secondary, more intimate attachment. Similarly, bacteria of the same or different species can adhere to each other and form a tight connection, a trait required for DNA-DNA transfer (mating). Tight pilus-induced cell-cell interactions of bacterial cultures (autoaggregates) also allow the cells to withstand stress. Finally, IV pilus retraction is also beneficial for DNA and bacteriophage uptake, as it allows for filament bound DNA or phages to be transported to cytoplasmic membrane-associated receptors.

Type IV pilin-like structures

Recently it has become apparent that type IV pili are a subset of a large and widely distributed system of cell surface complexes that are involved in processes such as, motility, macromolecular transport and adhesion, in gram-negative and gram positive bacteria as well as in archaea (28).

Pseudopili: The type II secretion system in gram negative bacteria transports proteins from the periplasm through the outer membrane(19). The transport of proteins like the *Klebsiella oxytoca* starch-degrading enzyme pullulanase (PulA) through the outer membrane not only requires an oligomeric secretin (PulD), but also involves 5 proteins that resemble type IV pilin-like proteins (pseudopilins)(37). They share sequence conservation in the N-terminal 30 amino acids, are processed by a type IV prepilin peptidase, and PulG, the most abundant pseudopilin, has a similar crystallographic structure to type IV pilins and forms pilus-like bundles when overexpressed in combination with the other *pul* secretion genes in *Escherichia coli*. These pseudopili, which have also been observed in *B. subtilis(12)*, are thought to form a piston that pushes the protein to the outer membrane. While the function is distinct from adherence, assembly and disassembly of the filament can also accomplish it.

Consistent with a similar assembly model as in type IV pili, pseudopilins are also co-transcribed with genes encoding PilB and PilC like proteins. While no PilT-like protein is required, it is possible that the PilB homolog provides the energy for both extension and retraction of the structure.

Archaeal flagella: Like bacteria, many archaea are motile by means of rotating flagellar filaments. However, in contrast to bacterial flagellar subunits, which are translocated using a specialized type III secretion apparatus, the secretion and assembly of archaeal flagellins resembles that of bacterial type IV pilins. They possess class III signal peptides that are cleaved by a prepilin-peptidase homolog (FlaK) before the incorporation of the protein into the flagellar filament. Moreover, like type IV pili, the filament is generally composed of multiple flagellins, that are present in drastically different stoichiometries. Finally, several components required for archaeal flagella assembly are related to those of the type IV pilus biogenesis system, including next to FlaK, a PilB ATPase homolog, FlaI, and the multispanning membrane protein FlaJ, which shows homology to PilC and might in a similar way serve as an assembly platform for the flagellum (Fig: 3B) (52, 64). Thus, this flagellum is likely assembled in a similar fashion as type IV pili.

Sulfolobus solfataricus substrate binding proteins: Albers et al. recently revealed that precursors of certain membrane-bound substrate-binding proteins (SBPs) also contain class III signal peptides that are cleaved by the preflagellin peptidase PibD (3, 4). Moreover, the deletion of S. solfataricus homologs of pilB or pilC results in impaired glucose uptake (71). As in type IV pilins, the class III signal peptides of SBPs may be necessary for the assembly of the proposed surface structure, the bindosome. The function of these structures may be to increase the local concentration of substrates, perhaps facilitating a more efficient uptake of nutrients (71). Although bindosomes appear to use the same assembly machinery as type IV pili, substrate-binding proteins are significantly larger than type IV pilins.

Archaeal non-flagellin type IV pilin-like subunits: Intrigued by the identification of subunits (SBPs) and structures (bindosomes) that are processed and assembled in a manner similar to type IV pili, we sought to determine the prevalence and diversity of archaeal proteins having similarities to type IV pilins**.** Using a PERL program (FlaFind) developed in our lab to perform a comprehensive analysis of 22 sequenced archaeal genomes, we identified a large number of archaeal genes containing 5' sequences that encode predicted class III signal peptides (63). Similar to genes that encode type IV pilins, many of

these genes are in operons that also contain homologs of the pilin-biosynthesis genes pilB and pilC. Interestingly, we demonstrated that a subset of the proteins containing a PFAM domain of unknown function (Duf369), located proximal to the N-terminus of the mature protein was specifically processed by a novel euryarchaeal prepilin-peptidase (EppA) homolog (57). In fact, recent studies by Wang et al. showed that Duf361-containing proteins of Methanococcus maripaludis indeed form pili. This first report of an archaeal pilus structure not only confirmed the presence of the predicted alpha-helical core, but also revealed a highly unusual and unique 3D structure (63). Finally, Albers et al. have confirmed our prediction based on in silico data that a set of S. solfataricus genes encodes pilin-like proteins that form surface structures (Albers, personal communication). The functional roles played by these structures are not yet clear.

Biofilms

In gram-negative bacteria, pili play important functional roles in several cellular processes that are necessary to establish and maintain biofilms, heterogeneous, morphologically variable structures composed of an extensive exocellular polymeric matrix consisting primarily of

polysaccharides. The benefits bestowed upon the bacteria that inhabit biofilms include an increased tolerance to environmental stresses, resistance to desiccation, and protection from toxins, including antibiotics and detergents, as well as metabolic synergies between and within species. Biofilms can damage industrial equipment, clog water pipes, contaminate food, and negatively impact human health. However, biofilms can also have beneficial impacts on the environment. They can facilitate the bioremediation of hazardous waste, the biofiltration of industrial wastewater, and the treatment of sewage and even garbage slurries. They also play important roles in removing pollutants from streams and estuaries. Although the vast majority of studies on biofilm formation have focused on gram-negative bacteria, biofilms in natural environments are also inhabited by single cell eukaryotes and archaea(1, 49, 60).

Even if archaea play profound roles in essential environmental processes and accumulating evidence indicates that they can have significant impacts on human health, we have only few data on the molecular mechanisms underlying most archaeal cellular processes. For example, while it is known that archaea inhabit biofilms, complex mucilaginous structures that provide resistance to a variety of environmental stresses in ecosystems ranging from dental plaque to hydrothermal vents, nothing is known about the cellular processes

used by archaea to establish or inhabit biofilms (18, 27, 30, 54, 55, 66). The incorporation of gram-negative bacteria into a biofilm requires that cells can adhere to surfaces, are motile along surfaces, and can autoaggregate (23, 26, 31). All these processes involve type IV pili, protein complexes that are members of a large set of widely distributed prokaryotic cell surface structures involved in such disparate and often essential processes as protein transport, DNA transfer natural competence and swimming motility (9, 13, 50, 53).

MATERIALS AND METHODS

Growth conditions

All strains were grown at 45 ^{o}C in complex media (Cx) or defined media (Hv-CA), liquid or 1.5 % agar plates (16).

H53 strain, used during the experiment, was supplemented with trypthophan and uracil in Ca media, with a final concentration of 50 μg/ml.

Proteins expression, extraction and western blotting

To investigate protein expression, we expressed the protein of interest with a C-terminal His-tag® and cloned them into the p-RV1-ptna plasmid (40). Then the plasmid was transformed into *H.volcanii* H53 strain following the standard PEG-mediated transformation of haloarchaea (16). The plasmid is inducible with tryptophan, so for this experiment, we added 100μg/ml (final concentration) of tryptophan to CX media.

Liquid cultures of H53 were grown to different phases, according to the microarray data. In particular for study on the expression of protein Hvo_A0632 and protein Hvo_02451 cultures were grown until mid-log phase (optical density at OD_{600}>0.6), for protein Hvo_02288 cultures were grown until stationary phase (optical density OD_{600}>0.8). The cells were then collected by centrifugation at 4,300 x *g* for 10 min at 4 ^{o}C. The cell pellets were washed once in PB(2M)S (2.14 M NaCl, 2.68 mM KCl, 10.14 mM Na_2HPO_4 and 1.76 mM KH_2PO_4, pH 7.4), with EDTA at final concentration of 10 mM, pelleted again and resuspended in 1/10th volume of PB(2M)S. Freezing in liquid nitrogen and thawing them at 37 ^{o}C for 3 times was the technique used to disrupted those samples. Insoluble debris was pelleted by three centrifugation at 5200 x *g* for 10 min at 4 ^{o}C. The samples were then fractionated by ultracentrifugation at 314,000 x *g* for 30 min at 4 ^{o}C. The supernatant (cytoplasmic fraction) and pellet (membrane fraction) were separated and the pellet were resuspended in 1 ml PB(2M)S. Then both samples were ultracentrifuged again at 314,000 x *g* for 30 min at 4 ^{o}C to remove any residual contamination. Cytoplasmic proteins were then precipitated using cold trichloroacetic acid (10% vol/vol) and washed in cold acetone (80% vol/vol).

All protein samples were stored in 1 NuPAGE lithium dodecyl sulfate sample buffer and supplemented with 50 mM dithiothreitol. Samples

were run on Bis-Tris NuPAGE gels (Invitrogen ®) under denaturing conditions using morpholinepropanesulfonic acid (MOPS). Proteins were transferred to polyvinylidene difluoride using the Bio-Rad Transblot-SD semidry transfer cell at 15 V for 30 min. Three buffers were used in semidry transfer: anode I (300 mM Tris, 10% [vol/vol] methanol, pH 10.4), anode II (25 mM Tris, 10% [vol/vol] methanol, pH 10.4), and cathode (25 mM Tris, 40 mM glycine, and 10% [vol/vol] methanol, pH 9.4). The membrane was probed with the primary antibody, Penta-His (1:1000) and the secondary antibody anti-mouse immunoglobulin (1:10000). Detection was performed using the Amersham ™ ECL Plus Western Blotting Detection System.

Trasmission electron microscopy

H. Volcanii cells grown on agar plates were scraped from the plates and were suspended in SMT buffer (3.5 M NaCl, 0.1 M $MgSO_4$ and 1mM tris-HCl, pH 7.2.) (57); 100 μl of suspension was mounted on cover slips coated with polylysine. After 1 hour of incubation at room temperature, excess cells were removed and replaced with 100μl of 2% glutaraldehyde in SMT. The preparations were incubated for 1 hour at room temperature and then washed with water. The fixed cells were

dehydrated through a gradient series of ethanol-water and ethanol-Freon TF and then coated with carbon. A Jeol 1200 electron microscope was used for examining the cells.

Twithching motility assay

To test for twitching motility single colonies were stabbed all the way down to the bottom of a plate, containing CX media at 1.5% agar concentration. Plates were incubated at 45 ^{o}C and checked every day.

Biofilm assay

Biofilm assay was modified from the ALI assay protocol(51) as follows: 3 ml of liquid culture in CX media, at ~600 nm of optical density, were incubated in each well of a 12-well plate. Glass coverslips (25x25, 0.19-0.25mm thick) were inserted in the well, with an angle of 90 degree. The plates, with the lid place on top and wet papers on the bottom were wrapped with polyvinyl paper to avoid evaporation of medium, and then incubated at 45 ^{o}C.

After different time intervals coverslips were removed from the wells with forceps and submerged for 3 minutes in 2% acetic acid, and then allowed to air dry. When the coverslips were completely dry they were stained by submersion in 0.1 % crystal violet for 5 minutes. The coverlisps were then washed 3 times with distilled water or until macroscopic trace of crystal violet disappeared. Dried coverslips were then observed under light microscope.

Autoaggregation assays

To test for autoaggregation 3 ml of liquid culture in Hv-CA media, at OD_{600} ~0.3, were incubated in each well of a 12-well plate. To test the effect of calcium on autoaggregation, different concentration of $CaCl_2$ were added to the coltures. $MgCl_2$ was used as well to test the effect of another divalent cation. Finally Hv-CA media was incubated with $CaCl_2$, to control unspecific precipitations. The plates were incubated at 45 ^{o}C and checked at different time intervals.

RESULTS

In silico analyses of putative pilin genes

FlaFind, the Perl program developed in the Pohlschröder lab., was used to indentify proteins with predicted class III signal peptide. FlaFind identified 36 substrates, more than in any other haloarchaeon tested thus far. Of these genes, 15 encoding FlaFind positives were co-transcribed with genes encoding PilB and PilC homologs (table 1). Moreover 6 predicted structural subunit contained a highly conserved PFAM domain of unknown function, Duf1628 (Fig. 4). Interestingly, the domain was in the class III signal peptide starting at the predicted SPase III cleavage site. Some of them were co-transcribed and exhibited homology to each other. However the other FlaFind positives showed neither a significant homology to each other nor to other genes with known function. The only exceptions were the flagellins. This suggests that they are involved in different functions.

FlaFind positives have distinct expression patterns

Microarray data was obtained from Dr. Charles Daniels, Ohio State University. *H. volcanii* cells were grown in complex (CX) or defined (CDM) medium with different NaCl concentration. These analyses revealed differential expression patterns for the *H. volcanii* genes that encode putative cell surface structural subunits (Table 1). Interestingly, several FlaFind positives are highly induced in CX medium, including Hvo_02450 and Hvo_02451. Conversely, operon Hvo_02385 - Hvo_02388 is not expressed or is expressed at extremely low levels during mid-log phase in either media; however, expression increases significantly during stationary phase. This, as well as the large size of these FlaFind positives, which encode proteins containing about 500 amino acids rather than the 150 amino acids typically encoded by other FlaFind positives, strongly suggests a unique role for these proteins. No significant expression was detected under any of the tested conditions for the operons Hvo_00620 – Hvo_00614 and Hvo_01160 – Hvo_01154. However homologs of Hvo_00618 in *Halobacterium NRC-1* are expressed under oxygen stress (N.Baliga personal communication). This data strongly suggest that the diverse structures are involved in distinct functions.

Consistent with the lack of sequence homology among the FlaFind positives, transcriptional analysis of *H.Volcanii* revealed distinct transcription patterns.

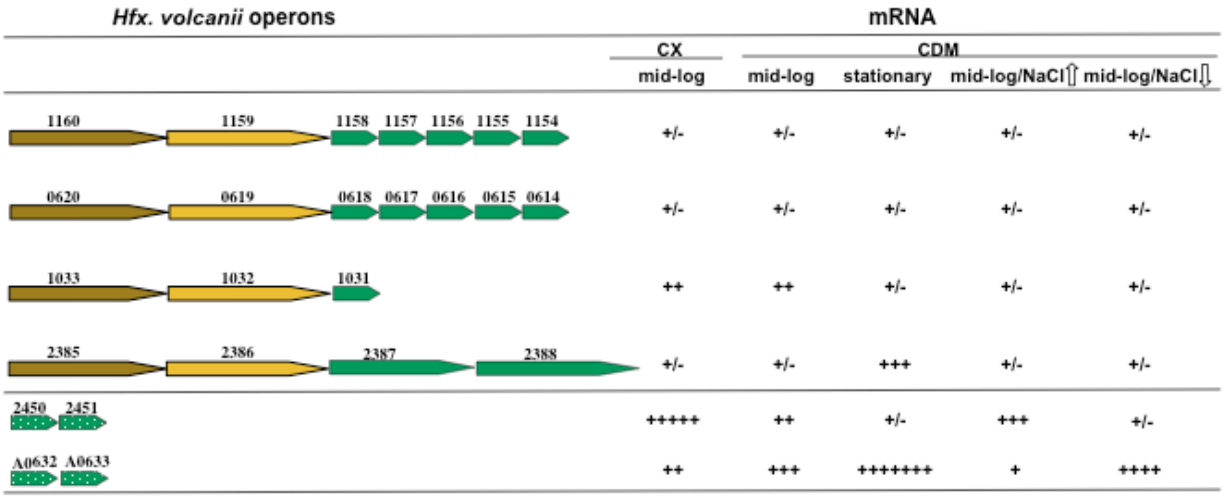

Table 1. *H. volcanii cells were grown in complex (CX) or defined (CDM) medium with optimal (2.2M) or 4.3M (up arrow), and 1.8M (down arrow) NaCl concentration. Brown arrows represent PilB homologs; yellow arrows represent PilC homologs; green arrows represent FlaFind positives; green arrows with dots represent FlaFind positives with Duf1628-domain. Numbers correspond to H. volcanii open reading frame (Hvo) numbers (1160 = Hvo_01160). In this table are only shown the FlaFind + that are co-transcribed with pili byosintesys genes or with other FlaFind +. Flagellins are not shown.*

We have also identified six *H. volcanii* genes that encode pilin-like proteins containing a conserved archaeal domain of unknown function (Duf1628) in operons lacking *pilB* or *pilC* homologs. Similar to Duf369 (63), Duf1628 begins at the predicted prepilin peptidase cleavage site of these proteins (Fig. 5). In fact, the most striking conservation of this domain is found in the hydrophobic stretch of the signal peptide, further supporting the conclusion that these genes encode class III

rather than class I or II signal peptide, as the hydrophobic stretches in class I/II signal peptides are not conserved, suggesting that the hydrophobic portion of class III signal peptides have a function in addition to SRP recognition. (e.g. provide scaffold for assembly of pili). Interestingly, the *Hbt. sp*. NRC-1 homolog of Hvo_02450 and Hvo_02451 (*H. volcanii* paralog, VNG6441h, Fig. 6) are predicted to contain a DNA-binding domain (N. Baliga personal communication). Therefore, it is possible that these proteins may be involved in DNA uptake, similar to the Com pseudopilin subunits found in gram-positive bacteria(12).

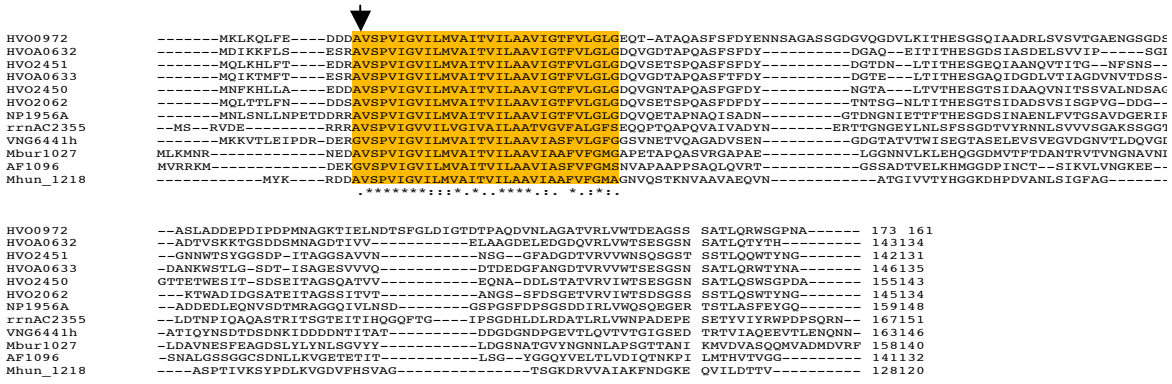

Figure 4. Amino acid sequence alignment of representative euryarchaeal proteins containing Duf1628 domains. *Amino acid sequences were aligned using ClustalW. Identity between the two sequences is indicted by one star, whereas strong and weak similarities are indicated by two dots and one dot, respectively. The hydrophobic region of the signal peptide is highlighted. Note that the domain of unknown function starts at the predicted prepilin peptidase cleavage site (arrow). (HVO: H. volcanii; NP: Natronomonas pharaonis; rrn: Haloarcula marismortui; VNG, Hbn. NRC-1; Mbur, Methanococcoides burtonii; AF, Archaeoglobus fulgidus; Mhun, Methanospirillum hungatei).*

Protein expression

We have attempted to overexpress each subunit FlaFind positive, that was ether co-transcribed with biosynthesis genes or other FlaFind positives, with a C-terminal his-tag under the control of an inducible *trp* promoter (40). Of the fourteen genes that were successfully cloned, Western blot analysis revealed that six expressed stable proteins. Interestingly, the genes expressing stable proteins (for Hvo_A0632, Hvo_A0633, Hvo_02388, Hvo_02450, Hvo_02451 and Hvo_01031) were also detected by microarray transcript analyses, perhaps indicating that these putative pilin-like proteins are only stable when incorporated into a cell surface structure. As expected for proteins incorporated into a membrane-anchored surface structure, each pilin-like protein we have tested thus far (Hvo_02450, Hvo_A0632, and Hvo_02388) is found primarily in membrane-associated protein fractions (Fig. 5).

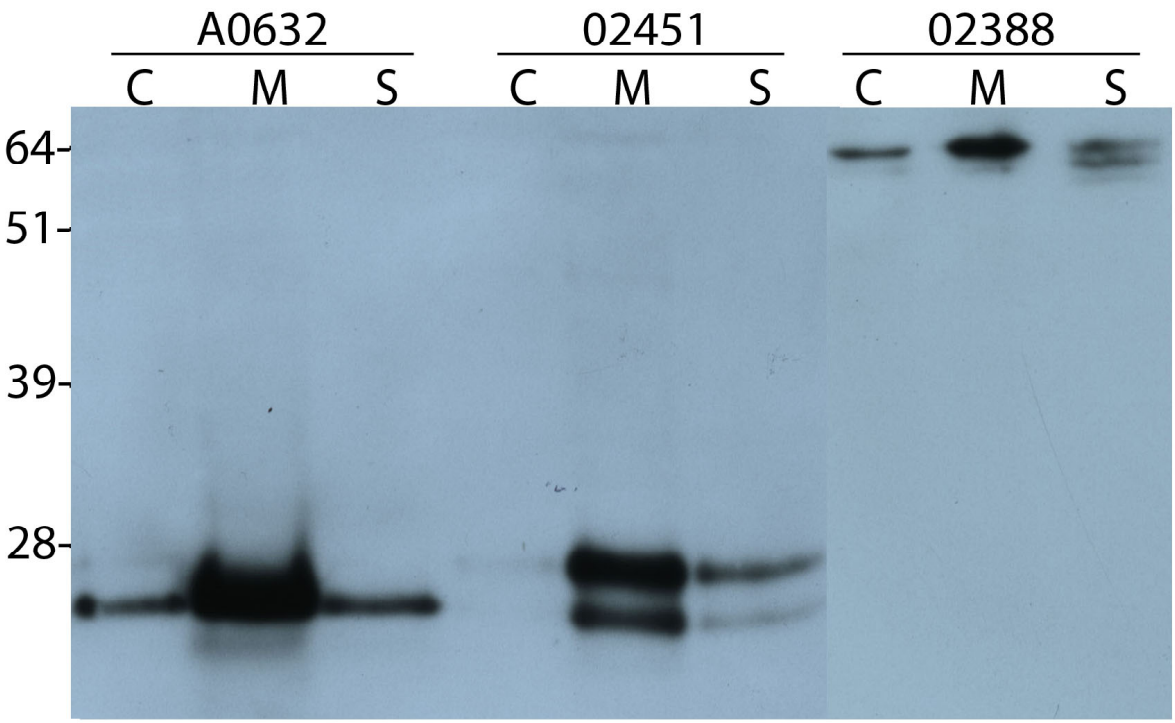

Figure 5. Putative subunits of *H. volcanii* surface structures are membrane-associated. *Anti-his antibodies were used in Western blot analysis of cytoplasmic (c), membrane (m), and culture medium (s) protein fractions from H. volcanii expressing his-tagged FlaFind positives. Molecular weight (Mr)-sizes are indicated on the left. (A0632-02451 provided by Kristin Toscano)*

H. volcanii **expresses surface structures**

In silico data suggest there should be surface structures present in *H.volcanii.* Electron microscopy of *H.volcanii* was only tested for conjugation, and the few data and micrographs that we have are dated 1989 (57).

Transmission electron microscopy (TEM) images of glutaraldehyde-fixed wild-type *H. volcanii* show different types of surface structure. We observed thin and long filaments, short thin filaments and short and thick filaments, which connected two cells together. This process is proposed to involve pili that provide close cell-cell connections resulting in the formation cytoplasmic bridges. TEM images showing such bridges are shown in Figure 6.

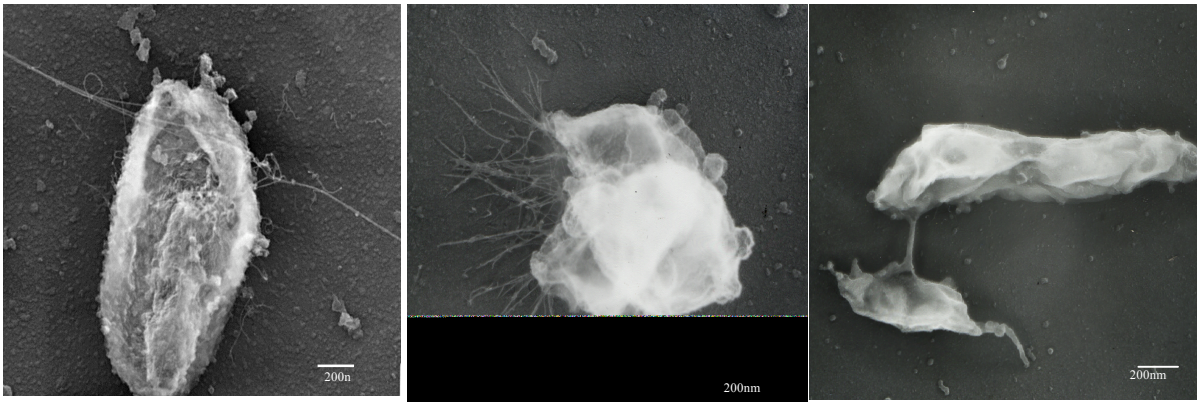

Figure 6. *H. volcanii* expresses flagella-independent surface structures*: Transmission electron microscopy (TEM) images of glutaraldehyde-fixed wild-type H. volcanii. On the right, H. volcanii can mate, which is proposed to involve pili that provide close cell-cell connections resulting in the formation cytoplasmic bridges(57).*

BACTERIAL TYPE IV PILI DEPENDENT CELLULAR PROCESSES DEMONSTRATED IN *H. VOLCANII*

Twitching motility

Even if in silico, in vivo and EM works suggest that *H. volcanii* presents diverse set of surface structures, it is not clear what they are involved in. As reported in the background section, in bacteria these structures are involved in different functions such as twitching motility, aggregation and biofilm formation, but beside being shown that H. volcanii can mate nothing was known about the other functions, and particularly nothing was known in archaea regarding twitching motility. Twitching motility, a mode of flagella-independent translocation across solid surfaces, relies on the extension, attachment and then retraction of type IV pili (42, 43). In bacteria, twitching motility is routinely assayed using the subsurface agar method in which the organism to be tested is stab-inoculated through a 1.5% agar plate. Twitching motility can be observed at the interstitial surface between the agar and the petri dish when an organism is capable of this type of translocation across surfaces.

We have shown that *H. volcanii* can move between the 1.5% agar and the plastic petri dish, forming a halo of growth around the stab that became visible after 3-4 days of incubation.

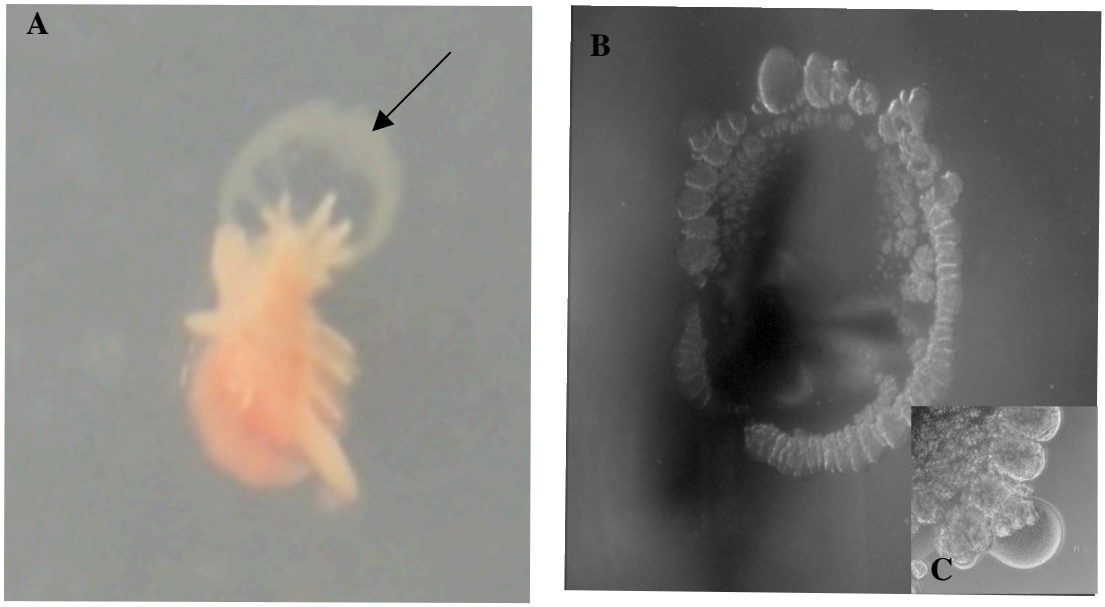

Figure 7: *H. volcanii* exhibits surface motility *(A). Inverted plates observation. Inverted microscopy of these halos reveals an increase in cell-density away from the stab (B) – the edges of the halos are not smooth, as would be expected for non-motile cells, but instead have a rough rippled appearance, suggesting that they can indeed move by twitching motility (C).*

We have shown that *H. volcanii* was able to move between the 1.5% agar and the plastic petri dish, forming a halo of growth around the stab. Confirmation of *H. volcanii* twitching motility will depend upon identifying the specific cell surface structure(s) necessary to form these halos. The dependence of *H. volcanii* twitching motility on pilus-

like surface filaments would require that the pili extend and retract. This is intriguing, as the *H. volcanii* genome does not contain an obvious PilT homolog. While it has been proposed that the extension and retraction of *K. oxytoca* and *B. subtilis* pseudopili occur in the absence of a PilT homolog, retraction of these structures has not been directly observed. Therefore, the identification of *H. volcanii* pili involved in twitching motility may provide the first opportunity to clearly demonstrate that pili can retract in an organism that lacks a PilT homolog.

Surface Adhesion

16S ribosomal RNA analysis has revealed the presence of archaea in complex biofilms. Consistent with this observation, *Archaeoglobus fulgidus* and *Pyrococcus furiosus* adhere to a variety of surfaces (27, 47, 61). However, the molecular mechanism(s) allowing surface adhesion by these archaea is unknown. Although we have identified putative type IV pilins in both *A. fulgidus* and *P. furiosus*, (57) the genetic tools necessary to determine their possible involvement in biofilm formation are lacking. Therefore, we determined whether *H. volcanii* could also adhere to surfaces using the Air-Liquid Interface (Ali) adhesion assay (51). We inserted glass microscope cover slips

into polystyrene wells containing *H. volcanii* mid-log phase cultures, and adapted existing protocols to determine whether surface adhesion by this haloarchaeon could be observed. We observed *H. volcanii* adherence to the cover slip at the air-liquid interface within 10 minutes of incubation and the density of cell attachment to the cover slip continued to increase during the next two hours (Fig. 8A). Interestingly, we also occasionally observed cell aggregates surrounded by a putative glycocalyx layer, which is reminiscent of the microcolonies found in biofilms (Fig. 8B).

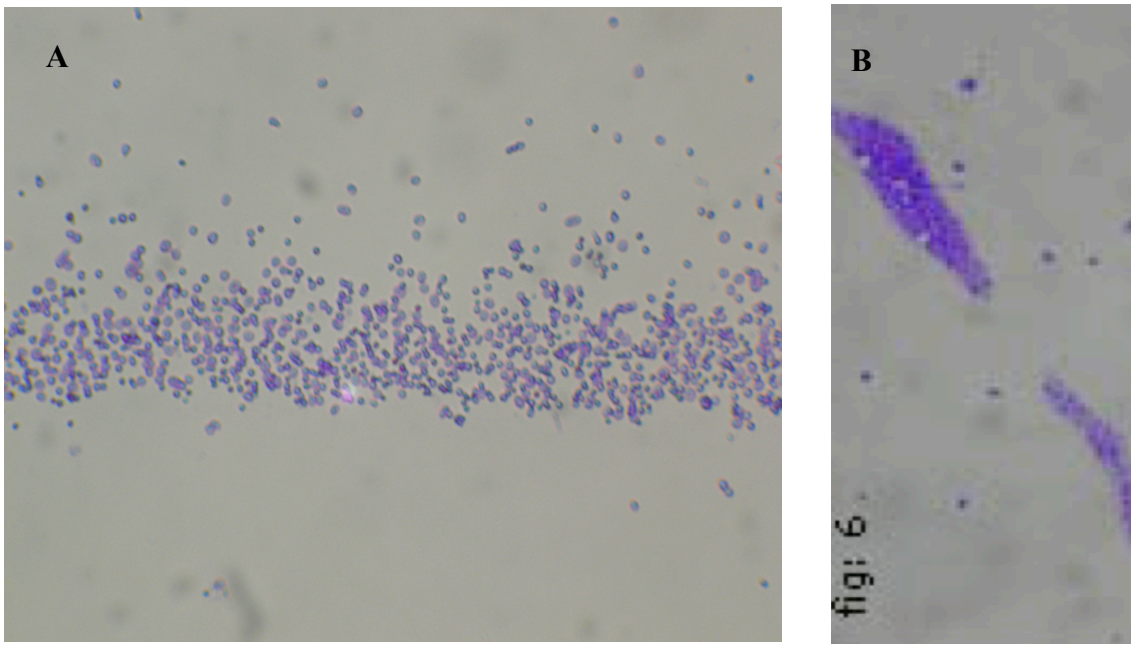

Figure 8. *H. volcanii* adheres to glass and autoaggregates. *H. volcanii adhesion to glass cover slips visualized by light microscopy of acetic acid fixed H. volcanii stained with 0.2% crystal violet (A). Occasionally, glycocalyx-like structures are observed (B).*

Autoaggregation

Autoaggregation can allow cells to withstand stressful condition. It also brings cells close to each other possibly allowing for DNA transfer that could provide more fitness by recombination.

Limited cell-cell interactions (autoaggregation) have previously been reported for *H. volcanii* grown on complex medium (32). We have now defined media conditions that allow highly efficient autoaggregation of *H. volcanii* (Fig. 9). While we have not observed autoaggregation in complex or defined medium lacking the divalent cation Ca^{++}, efficient autoaggregation was observed in defined medium containing Ca^{++}, but not Mg^{++} (Fig. 9). This assay will now allow us to determine whether the putative type IV structural subunits are required for these cell-cell interactions.

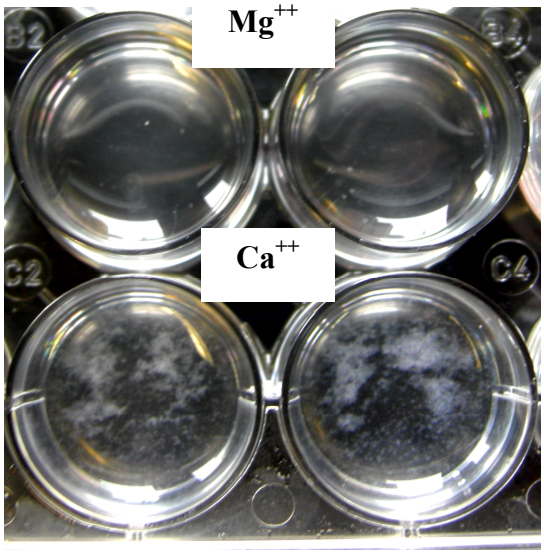

Fig. 9. *H. volcanii autoaggregation in the presence of Ca^{++}, but not Mg^{++}.*

CONCLUSIONS AND FUTURE DIRECTIONS

- Putative subunits of archaeal type IV pili-like structures were identified, and the differential expression and sequence divergence, suggest different functions for several of these predicted pili.
- An EM protocol that allows us to see *H. volcanii* pilin-like surface structures was developed together with methods for biofilm assay and autoaggregation to be used at high concentration of salt.
- With this work we determined that *H. volcanii* can autoaggregate with calcium mediation and form biofilm.
- An important result is the fact that for the first time *H. volcanii* was shown to exhibit twitching motility, a surface motility that had thus far not been reported for archaea.

Development of assays that demonstrated autoaggregation, twitching motility and adhesion will now be useful to determine the involvement of specific surface structures in these functions.

- ◎ Rising antibody specific against subunit of predicted structures and development of protocol for immunogold-labeling will be used to identify specific structures.
- ◎ The generation of mutants for these structures to be tested with these assays and under EM, will be another important step to better understand the functions and processes in which these structures are involved.
- ◎ Stress conditions, like UV exposure and H_2O_2, will be used to test for expression and functions of the surface structures that were not expressed under condition tested thus far.

ACKNOWLEDGMENTS

Thank to Dr. Tatyana Svitkina (Department of Biology University of Pennsylvania, Philadelphia) for skillful assistance in Electron Microcopy.

Thank to Enrico Perrotta ("A.Saita" Electron Microscopy Laboratories-Università della Calabria, Cosenza, Italy) for the Electron Microscopy assistance.

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