GENETIC RELATIONSHIPS AND THE EFFECTS OF AN ANTIMICROBIAL AGENT ON THE HYPERTHERMOPHILE *PYROCOCCUS FURIOSUS*

by

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(Under the Direction of MICHAEL W. W. ADAMS)

ABSTRACT

The goals of this research were to develop whole genome microarray technology for the hyperthermophilic archaeon, *Pyrococcus furiosus*, and to use this approach to a) evaluate genetic relationships between *P. furiosus* and *P. woesei,* and b) determine the mechanism of action of the an antimicrobial agent, Roussin's Black Salt (RBS) on *P. furiosus*. Microarrays have been demonstrated to be one of the most powerful molecular biology techniques and allow the rapid analysis of whole genomic transcript responses of organisms to metabolic stresses. The processing and biochemical techniques involved with microarray have undergone many revisions and improvements as innovative products are made available. Genomic comparisons between *P. furiosus* and *P. woesei* were made possible by advances in microarray design. It was shown that of the 2,192 annotated open reading frames (ORFs) in the *P. furiosus* genome, at least 104 of them were absent from the genome of *P. woesei*. These "missing" ORFs were arranged in distinct groups within the *P. furiosus* genome and flanked by insertion sequences which are believed to be involved with lateral gene transfer (LGT). The likely mechanism of LGT was

derived from sequence analysis of the two genomes, and it is concluded that *P. furiosus* is a sister or parent strain of *P. woesei*.

RBS is a broad spectrum bactericide that has proven very effective in controlling pathogenic anaerobes such as *Clostridium* both in the vegetative and spore state. RBS has the formula $[Fe_4S_3(NO)_7]$ and it has been generally assumed that the release of nitric oxide (NO) is the cause of the cytotoxic effects although the mechanism is unknown. The effects of NO release were to be assessed by DNA microarray analyses. However, it was demonstrated using growth studies, membrane analyses, and scanning electron microscopy that NO does not play a role; rather, the mechanism of toxicity involves membrane disruption. Moreover, insoluble elemental sulfur, which is reduced by *P. furiosus* to hydrogen sulfide, prevents membrane disruption by RBS. RBS therefore appears to be a novel type of inorganic surfactant, and its mechanism is independent of NO and involves membrane specific disruption.

INDEX WORDS: Hyperthermophile, Roussin's Black Salt, *Pyrococcus furiosus*, Microarray, *Pyrococcus woesei*, Inorganic-surfactant, Genomic comparisons, Insertion sequences

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DEDICATIONS

My parents, David and Terry Brehm, have supported me throughout the years and have always provided good advice to face life's challenges and aspire to do great things.

My wife Anne Marie, who I met while learning dancing, who patiently and lovingly watched over and took care of me knowing first hand the stress involved from pursuing her own Ph.D degree in Linguistics.

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I wish to follow up my acknowledgements with a quote. Even though my mind is passionate for the sciences, my heart embraces literature. Out of the many quotes I have collected this paraphrased quote from a speech made by Dr. Albert Einstein in 1918 at Dr. Max Plank's birthday party gave me strength to continue through the years in graduate school:

"In the temple of science there are many mansions and various indeed are they that dwell therein and the motives that have led them there. Many take to science out of a joyful sense of superior intellectual power; science is their own special sport to which they look for vivid experience and the satisfaction of ambition; many others are to be found in the temple who have offered the products of their brains on this altar for purely utilitarian purposes. Were an angel of the Lord to come and drive all the people belonging to these two categories out of the temple, it would be noticeably emptier but there would still be men and women of both present and past times left inside.

If the types we have just expelled were the only types there were, the temple would never have existed any more than one can have a wood consisting of nothing but creepers. Those who have found favor with the angel are somewhat odd, uncommunicative, solitary fellows, really less like each other than the hosts of the rejected. What has brought them to the temple no single answer will cover: Escape from everyday life, with its painful crudity and hopeless dreariness, from the fetters of one's own shifting desires, a finely tempered nature longs to escape from the noisy cramped surroundings into the silence of the high mountains where the eye ranges freely through the still pure air and fondly traces out the restful contours apparently built for eternity."

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LIST OF ABBREVIATIONS

CMC - critical micelle concentration

HLB - hydrophile-lipophile balance

LUCA – Last universal common ancestor

NADPH – nicotinamide adenine dinucleotide phosphate (reduced)

NADH – Nicotinamide adenine dinucleotide (reduced)

NO – Nitric oxide

RBS – Roussin's Black Salt

SDS – sodium dodecyl sulfate

SCC – sodium chloride citrate buffer

CHAPTER 1

INTRODUCTION

Only in the last 50 years were communities of organisms discovered living in environments at extremes of pH, temperature, and salinity. The diversity of organisms on Earth had originally been placed within a phylogentic tree that contained two domains of life identified as Eukarya (true nucleus) and Eubacteria (true bacteria) (17). Through 16S rRNA sequencing a third domain of life termed the Archaea (the ancients), were identified through the pioneering work of Dr. Carl Woese (20, 80). Archaea are best known for inhabiting extreme environments on Earth from the salt saturated waters of the Dead Sea, the frozen wastelands of the artic, and five kilometers under the seas living next to volcanic chimneys devoid of sunlight. The classification of Archaea is composed of halophiles (salt-loving), psychrophiles (cold-loving), acidophiles (acid-loving), alkaliphiles (base-loving), methanogens (methane-producing) and thermophiles (heat-loving). The archaea are organized into four sub-domains (see Figure 1.1): crenarchaeota (thermophiles and acidophiles), euryarchaeota (halophiles and methanogens), nanoarchaeota (symbiotic thermophiles), and korarchaeota (as yet uncharacterized 16S environmental isolates) (10, 24, 43, 49, 81, 83). Two hundred fifty five archaea have now been reported in pure culture, and genome sequences have been reported for 47 of them (http://www.genomesonline.org, www.dsmz.de).

The thermophiles are mostly strict anaerobes and the slowest evolving organisms, occupying the lowest branches of the phylogenetic tree (79). This would suggest that the last universal common ancestor (LUCA) is also a hyperthermophile and evolved from a hot anaerobic environment (78). Such conditions were postulated to have been present shortly after the Earth was formed and the surface severely bombarded by meteorites (51, 52, 69). The LUCA formed on Earth in these extreme environments was likely a communal organism where separate species diverged only when a critical threshold of molecular complexity was reached (79). Thermophiles studied today have been found to occupy a wide range of temperatures up to 121ºC. Organisms that thrive in temperature ranges of 40–80°C are referred to as thermophiles, while those that grow in the temperature range of 80–121ºC are commonly referred to as hyperthermophiles. The highest temperature at which life has been reported to grow is 121°C, which is accredited to Strain 121 (37).

Although some hyperthemophilic oxygen-tolerant and oxygen-utilizing organisms can be found, see Table 1.1, the majority of hyperthermophiles are anaerobic. Presumably this is because at temperatures above 80°C oxygen solubility decreases. Respiration among hyperthermophiles is diverse, although many use carbohydrates or peptides as a source of carbon. Terminal electron acceptors include elemental sulfur, protons, and oxygen, forming end products of H_2S, H_2, and H_2O, respectively (28, 31). For over 15 years organisms from the phylum crenarchaeota and euryarchaeota have been studied extensively. One particular aspect about hyperthermophiles that has been studied is how structural organization of molecules is accomplished to withstand the temperatures greater than 80°C. Homeoviscous adaptation describes the changes in lipid profiles to adapt to environmental changes (27). The membrane is

an important structure providing an interface by which to regulate the exchange of nutrients, acquisition of electron sinks for energy production, environment sensing, osmotic pressure control, and uptake or distribution of genetic elements, just to name a few. The fluid mosaic model has been the standard for understanding mesophilic membrane structure using monopolar amphiphilic lipids consisting of glycerol molecules covalently attached by ester linkages to saturated or unsaturated hydrophobic fatty acids. As the temperature increases, the population of unsaturated lipids is decreased and the population of saturated lipids increases (21, 27). These molecule population changes modulate the membrane fluidity, which in turn allows the regulation and maintenance of the essential environmental interactions of the organism. While the fluid model is still applicable, the lipid structures found in mesophilic bacteria are inadequate to deal with the high temperatures in thermophilic environments (27). Archaeal lipid structure is distinctively different, utilizing ether linkages, lipid structures are predominantly a tetraether 'monolayer' with varying head groups usually unique to different taxonomic archaea groups. By mixing the populations of tetraether and diether lipids hyperthermophiles form a heterogeneous membrane structure which provides a physically high temperature stability not capable from monolayer structures (27). The lipid cores can be further stabilized against high temperatures modified by introducing cyclopentane moieties. Another membrane feature of archaea and bacteria is the single protein or glycoprotein structure known as the S-layer (61). As compared to membranes without an S-Layer, this structure provides enhanced stability while still providing access for the cell to the environment through repeating standard sized 2-8 nm pores (61). The unique structures of archaeal lipids have encouraged patents for what are hoped to be the next generation high temperature enduring, chemical resistant lubricants for applications in mechanical engineering (27).

Hyperthermophiles are found in marine and in freshwater thermal ecosystems. In both environments large volumes of water is heated by volcanic activity. Whether at a shallow pool, the shoreline of a volcano or five kilometers below the sea surface these locations are home to hyperthermophiles. The heat that drives these ecosystems comes from magma under the Earth's crust (54). The magma causes the Earth's crust to shift and move which in turn generates cracks and rifts due to these massive stresses. Whether on land or in the ocean, water seeps through the crust by these cracks reaching the magma heated rock below. The water is heated to temperatures in excess of 300°C. Minerals and gases are able to dissolve into the super heated water. Once heated the water begins to rise ejecting with force to the surface of the Earth. Depending on the environments where the water is ejected the dissolved materials will precipitate out of the water. In the ocean the near freezing water of the deep causes the minerals to precipitate manifesting as dark clouds of black 'smoke'. The chimney structures from which the water is ejected are called 'black smokers' (54, 70, 72). On land the heated water is ejected in geysers, depositing the minerals in pools and in mud calderas (48, 56).

The island of Vulcano, located west of Italy and north of Sicily, has proven to be a rich source of hyperthermophiles from both the archaeal and bacterial domains. The most extensively characterized hyperthermophilic archaeon isolated from Vulcano is *Pyrococcus furiosus*, which was isolated by Dr. Karl Stetter and coworkers (23). *P. furiosus* is a heterotrophic obligate anaerobe that can reduce protons or inorganic sulfur producing hydrogen and hydrogen sulfide, respectively (23). Carbon sources for growth include peptides and simple and complex sugars. The latter are fermented using a modified Embden-Meyerhof pathway

producing organic acids and CO_2 as products (32, 60). The genome of *P. furiosus* contains 2065 open reading frames (ORFs) approximately one third of which are homologous to genes of known function, one-third are conserved in other organisms but have limited functional information, and the function of the remaining third is completely unknown. *P. furiosus* has been extensively studied with respect to enzyme characterization, genomic comparisons, stress responses, metabolic characterization using biochemical and molecular biology techniques (62-64, 76).

Genetic systems of hyperthermophiles are beginning to be used in research but one of the major hurtles has been that antibiotics used in equivalent mesophilic systems are labile at high temperatures. While artificial genetic systems are particularly difficult to develop, in the natural environment genetic exchange appears to be prevalent, as shown by comparing genomes of hyperthermophiles; as yet however the mechanism of lateral gene transfer at 100°C is unclear. In the following sections part 1 covers antimicrobial molecules, part 2 explains what is known about lateral gene transfer among hyperthermophiles, and part 3 provides a history of the microarray technique.

1. Antimicrobial compounds

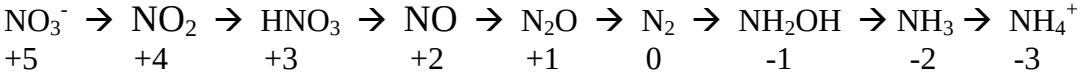
1A. Reactive Nitrogen Species

The nitrogen cycle describes the pivotal processes of nitrogen assimilation and dissimilation that all life forms on this planet take part in to meet metabolic needs for organic nitrogen. The major source of nitrogen is from the earth's atmosphere, diffusing into the soils where it is "fixed" by microbes into biologically available species. Nitrogen is essential to all

living organisms. However, some oxidized nitrogen forms are toxic to organisms and are known as reactive nitrogen species (RNS). For more than a thousand years, nitrite and nitrates have been used for the curing of meats and fish from spoilage-causing microbes, such as the anaerobic organisms belonging to the genera *Clostridium* (7, 13, 45, 50). From humankind's early history to today RNS has been used to preserve food the techniques have been refined although the mechanism of microbial inhibition remains largely unknown (7). In the 1970's, health concerns over possible connections between nitrite and cancer caused a steady decline in the amount of nitrite used in American and European foods (13, 65, 75, 77). Despite the active historical use of RNS, in the last fifty years research has yet to reveal the precise mechanism by which microbial growth is inhibited (7, 12, 13, 36). The potential health risks and antimicrobial effects associated with RNS demand that a comprehensive understanding of reaction mechanisms be achieved. Research involving archaea and nitrogen species involved assimilatory and dissimilatory nitrogen reactions, but the sensitive of archaea to RNS has not been reported (1, 12, 57, 66).

Nitrogen can occur in various oxidation states from -3 to +5 (see Scheme 1). The stable intermediates found in the nitrogen cycle are nitrate (NO_3^-), nitrite (NO_2^-), nitric acid (HNO_3), nitric oxide (NO), nitrous oxide (N_2O), nitrogen gas (N_2), hydroxylamine (NH_2OH), and ammonia (NH_3). The reductases needed to carry out these 1 or 2 electron step processes are associated with detoxification or energy metabolism.

Scheme 1



Nitrite and NO are known to be toxic to prokaryotes. The cytotoxic effects of nitrite occur by its rapid conversion to NO, which occurs spontaneously in acidic aqueous solutions under both aerobic and anaerobic conditions (50). NO is highly reactive and has been identified in eukaryotes as being involved with many cell functions from cell signaling between neurons to being used by macrophages as a bactericide. NO has a very short half life in biological systems, measured in seconds, as it readily reacts with primary thiols, amines, and metals (44, 86). The production of NO from unstable nitrite is not consistent with the observed enduring antimicrobial affects on food preservation, as the effects of NO would be depleted long before the antimicrobial effects are observed (50). One of the possible explanations is that NO reversibly reacts with primary thiols, amine groups, and iron, forming S-nitrosothiols, nitrosoamines, and iron nitrosyl, respectively (11, 85). These complexes act as storage molecules prolonging the life span of NO. Once NO is produced it is unclear if it causes the microbial inhibition through direct or indirect interactions.

RNS in the forms of nitrate and nitrite are present at shallow and deep thermal vents where hyperthermophiles are found (2, 3, 55). How hyperthermophiles metabolize compounds in the nitrogen cycle has been explored (12). Yet how RNS interacts with archaeal hyperthermophiles is unknown. In chapter 4 the effects of the putative NO generator known as Roussin's Black Salt is described.

2B. Antimicrobial Surfactants

Surfactants (sometimes known as detergents) are amphiphilic molecules that accumulate at the interface of immiscible hydrophobic and hydrophilic fluids, and lower the surface tension

thus increasing solubility of the two phases (73). There are two distinct classes of detergents organic and inorganic surfactants. Organic surfactants are molecules that typically contain carbon, nitrogen, oxygen, and hydrogen. Organic surfactants can either be produced from an organism or manufactured abiotically. Organisms use surfactants to help facilitate mobility, uptake of nutrients, cell signaling, and by lysing other cells or affecting spores killing dormant organisms to establish an advantage over competitors (73). Inorganic surfactants possess the same attributes as organic surfactants only that the molecular composition lacks carbon and they are not produced by organisms.

Surfactants that are classified as antimicrobial are amphiphilic molecules that cause membrane disruption of microbes leading to cell lyses (73). In hospitals, industry and private homes antimicrobial surfactants have been widely used. Similar to antibiotic resistance, microbes have developed different methods to survive the membrane disrupting effects of surfactants. Resistance to surfactants is possible through production of chelators that bind the amphiphilic molecule, modifying the lipid raft composition of the membrane, efficient efflux pumps that remove the molecule from the lipid layer and excretion of enzymes that can cleave the amphiphilic molecule (9, 33, 67, 73). The mechanism of surfactants and resistance is of great interest to public health research.

2. Lateral Gene Transfer

The phylogenetic tree may not be a tree at all, but rather a bramble of twisting and interconnected lineages, as pointed out by Doolittle (20). Genetic exchange that does not rely on vertical lineage has recently become widely accepted theory, exemplified by the growing

problem arising from antibiotic resistant strains of bacteria (22, 34, 71, 82). Known as horizontal or lateral gene transfer (HGT or LGT), it is the process of genes being transferred between organisms, sometimes across the domains of life, which is accomplished by one of three basic mechanisms. The first is conjugation, which involves the transfer of a plasmid by cellular contact, whereby the plasmid finally integrates itself into the new host conveying genes it carries. The second is transduction, whereby bacteriophages negotiate indirect exchange via transducing particles that erroneously package chromosomal DNA. The third mechanism involves transformation, where DNA is directly taken up by the recipient cell from the environment (71). Mobile elements, such as insertion sequences (IS) and transposons, have been implicated in transferring gene in LGT between organisms. IS elements consist of a coding region for the transposase and terminal inverted repeats; the inserted IS element is flanked by direct repeats of host DNA (Figure 1.2). Sometimes referred to as a selfish gene, transposons only code for a transcript that will produce an enzyme that catalyzes the transposition of the transposon into host DNA. In certain cases two identical IS can flank a set of DNA or an operon forming what is known as a composite transposon and can relocate the genes between the IS elements within the genome or possibly transport to another organism (Figure 1.3) (26).

Transposons are found throughout bacteria and archaea, including thermophiles. Although LGT events are suspected to happen at temperatures where thermophiles live, it is difficult to determine the tempo, mechanism or conditions when it does happen. The species of the genus *Pyrococcus* appear to have had considerable genomic rearrangements possibly due to IS events (41, 42, 87). *P. abyssi*, *P. furiosus* and *P. horikoshii* are found in different geographical locations on the planet and in different vent conditions in that *P. abyssi* and *P. horikoshii* are

found in deep vent communities whereas *P. furiosus* is found in shallow marine vents (15). Even though homology of the ORFs between these organisms is conserved across sections of the genome and even over gene cassettes, considerable genome rearrangements have occurred in the divergence of *Pyrococcus*. Organisms located geographically close to *P. furiosus* exhibit close homology and even identical ORFs across genera (19). In some cases insertion sequences are still found flanking the genetic sequences. Sequencing the genomes of organisms is the primary mechanism to compare their gene repertoires. However, as shown in Chapter 2, considerable insight can be gained in the absence of a genome sequence using DNA microarray technology.

3. DNA Microarray Technology

Southern's pioneering work using labeled nucleic acids as probes to hybridize to electrophoresed targets was a profound contribution to molecular biology (68). New techniques based on Southern blotting include what is known as DNA microarray technology. This has proven to be a tool that has powered a revolution in genome research, bioinformatics and industrial markets (6, 14, 40, 53, 58, 59, 64). Microarrays use the principal methodology of a Southern blot but with a high-through-put capability. The era of genome sequencing lent even more versatility and utility to DNA microarrays, as it allowed transcript expression of a whole genome. For the construction of microarrays, gene elements of choice in the form of oligonucleotides, PCR products or library clones, are covalently attached to a glass solid support using aminosilane or epoxysilane linkages in a coordinated matrix format. The RNA or DNA probe for the array is randomly labeled and is allowed to hybridize to the microarray matrix. Unhybridized probe is washed away and the remaining labeled probe that is complexed to the microarray elements is measured individually correlating to the relative amount of a particular

message (target) present. The DNA microarray approach that was developed for *P. furiosus* is discussed in detail in Chapter 2.

As mentioned above, in addition to their utility in measuring RNA transcripts, DNA microarrays can also be used for the direct comparison of the genomes of closely related organisms (18, 25, 46, 84). Homologous ORFs on the microarray will hybridize labeled complimentary genomic DNA which allows for the identification of similar genes between organisms. A limitation of this technique is that the microarray can only detect the presence or absence of ORFs on the slide; it cannot detect ORFs present in the test organism that are not on the slide. The sensitivity and quantization of microarray techniques have improved to the level of being able to detect point mutations within gene sequences (5). The plastic genomes of prokaryotes can be daunting to characterize, yet using genomic comparisons with microarrays, genetic drift, lateral gene transfer and evolutionary events can all be observed

.

4. Purpose of Research

The purpose of this research was to develop DNA microarray technology for *P. furiosus*, a hyperthermophilic archaeon whose genome has been sequenced. This would allow for high through put analysis of P. furiosus transcript regulation in response to environmental stresses. By monitoring transcriptional responses it becomes possible to understand the purpose of the ~1000 ORFs of unknown function in *P. furiosus*. The second goal was to utilize microarray techniques for comparative genomics to analyze a hyperthermophile closely related to *P. furiosus*. The ability to quickly assess similarities of genomic ORF compositions from among the hundreds of unsequenced organisms, and environmental samples, may prove to be the

solution to assessing the presence of desired homologs of ORFs without sequencing the whole organism. The third goal was to use the microarray technique to investigate the mechanism by which *P. furiosus* responds, at the transcript level, to the RNS and putative NO generator known as Roussin's Black Salt. The mechanism microbial inhibition due to NO is not known. By studying NO generators and metabolic responses to RNS it becomes possible to better understand the mechanism(s) involved that inhibit microbial growth.

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Table 1.1: Representative hyperthermophiles (≥80°C). Data adapted from (4, 29-31, 35, 38, 39, 47, 74).

Domain	Phylum	Genus	species	T_{max} (°C)	Electron donors	Electron acceptor
Archaea						
	Crenarchaeota					
		Acidianus	*ambivalens*	87°	H_2	S°, O_2
		Archaeoglobus	*fulgidus*	83°	H_2, organic molecules	SO_4^{2-}, $S_2O_3^{2-}$
		Hyperthermus	*butylicus*	100°	peptides	S° or H^+
		Pyrobaculum	*aerophilum*	100°	Peptone, Yeast, H_2	O_2, NO_2^-, NO_3^-
		Pyrodictium	*abyssi*	98°	H_2	S°, $S_2O_3^{2-}$
		Thermodiscus	*maritimus*	88°	yeast extract, H_2	H^+
		Thermosphaera	*aggregans*	85°	yeast extract, peptides	H^+
		Staphylothermus	*marinus*	85°	peptides	S°
		Sulfolobus	*solfataricus*	85°	Sugars, amino acids	O_2
	Euryarchaeota					
		Methanococcus	*jannaschii*	80°	H_2, formate	CO_2
		Pyrococcus	*furiosus*	100°	sugars, peptides	S° or H^+
			woesei	100°	sugars, peptides	S° or H^+
			horikoshii	100°	peptides	S°, H^+
			abyssi	100°	peptides	S°, H^+
		Thermococcus	*kodakaraensis*	85°	peptides, starch	S°, H^+
			litoralis	85°	sugars, peptides	S° or H^+
Bacteria						
		Aquifex	*aeolicus*	85°	H_2	H_2S, SO_4^{2-}
		Thermotoga	*maritima*	80°	Sugars	S° or H^+

Figure 1.1: Hyperthermophile phylogenetic tree. Modified from (8).

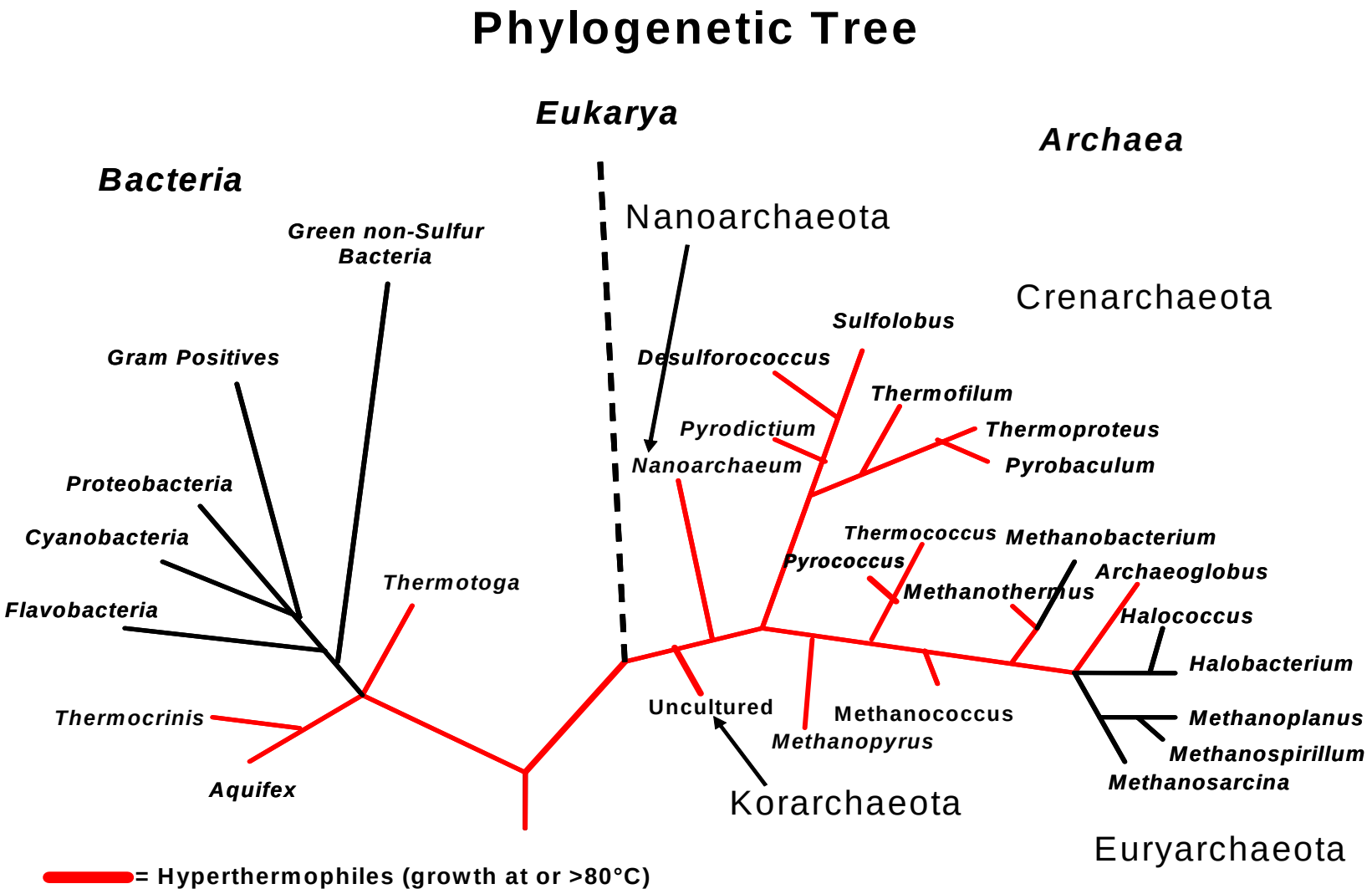


Figure 1.2: Anatomy of a *P. furiosus* ISPfu1 (IS6 family) insertion sequence. Adapted from (16, 19)**.**

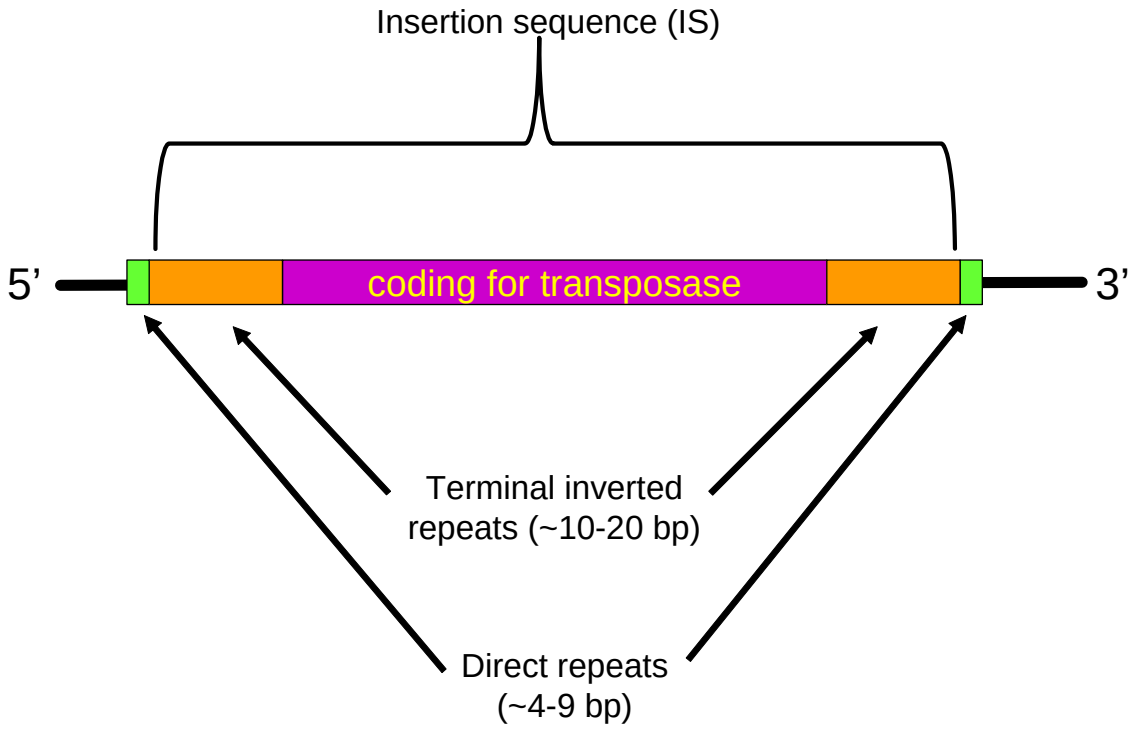
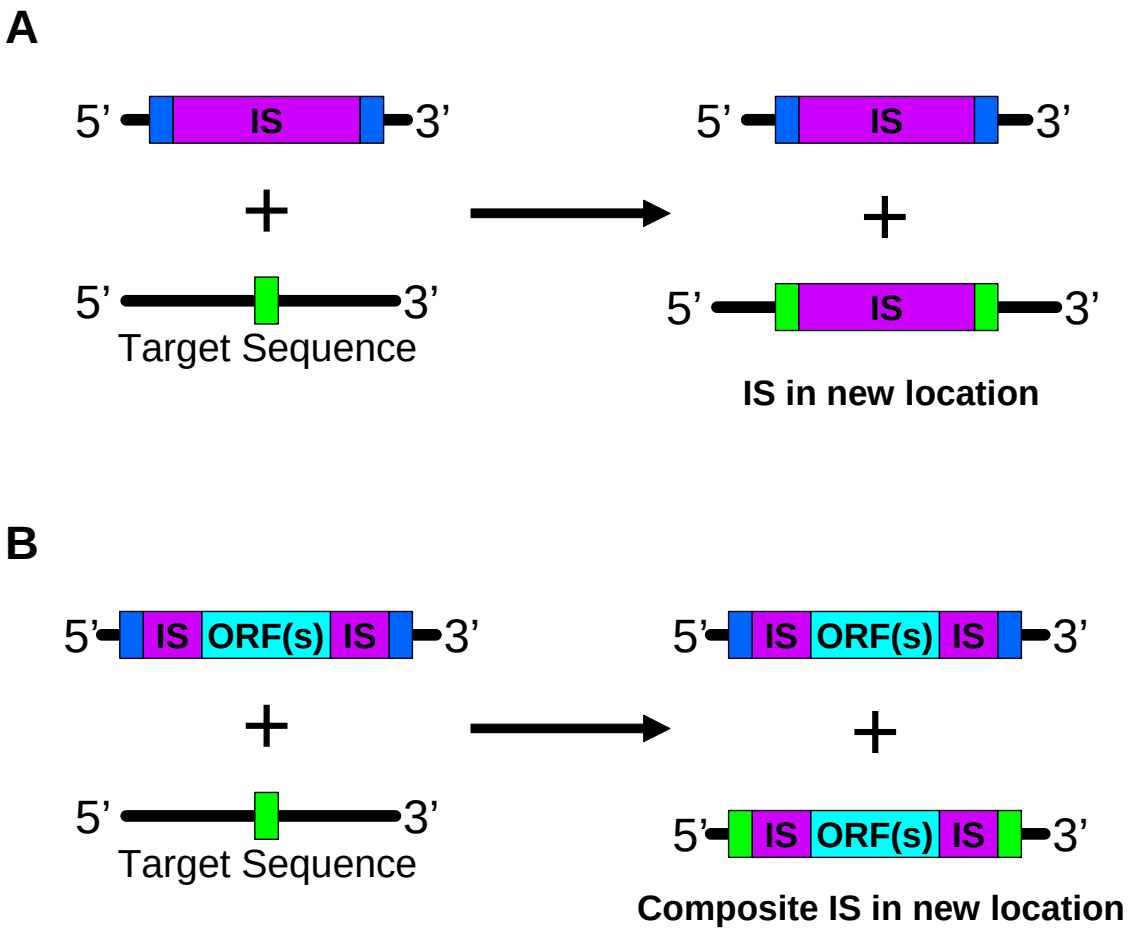


Figure 1.3: Proposed transposition scheme of an ISPfu1 insertion sequence. A. Replicative transposition of a single transposon. B. Transposition of a composite insertion sequence. Blue and green boxes represent different sequences of direct repeats. Purple boxes represent a *P. furiosus* insertion sequence. Adapted from (16).



CHAPTER 2

METABOLIC AND EVOLUTIONARY RELATIONSHIPS AMONG *PYROCOCCUS* SPECIES: GENETIC EXCHANGE WITHIN A HYDROTHERMAL VENT ENVIRONMENT

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Abbreviations: DR, direct repeat; IS, insertion sequence; LCTR, Long chain terminal repeats; ORF, open reading frame; PCR, polymerase chain reaction; RBS, Roussin's Black Salt.

Abstract

Pyrococcus furiosus and *P. woesei* grow optimally near 100 °C and were isolated from the same shallow marine volcanic vent system. Hybridization of genomic DNA from *P. woesei* to a DNA microarray containing all 2,065 open reading frames (ORFs) annotated in the *P. furiosus* genome, in combination with PCR analysis, indicated that homologs of 105 ORFs present in *P. furiosus* are absent from the uncharacterized genome of *P. woesei*. Pulsed field electrophoresis indicated that the two genomes are of comparable size and the results were consistent with *P. woesei* lacking the 105 ORFs found in *P. furiosus*. The missing ORFs are present in *P. furiosus* mainly in clusters. These include one cluster (Mal I, PF1737-PF1751) involved in maltose metabolism, and another (PF0691-PF0695) whose products are thought to remove toxic reactive nitrogen species. Accordingly, it was shown that *P. woesei,* in contrast to *P. furiosus,* is unable to utilize maltose as a carbon source for growth, and its growth (on starch) was inhibited by the addition of a nitric oxide generator. In *P. furiosus* the ORF clusters not present in *P. woesei* are bracketed by, or are in the vicinity of, insertion sequences (IS) or long cluster terminal repeats (LCTR). While the role of LCTRs in lateral gene transfer is not known, the Mal I cluster in *P. furiosus* is a composite transposon that undergoes replicative transposition. The same locus in *P. woesei* lacks any evidence of insertion activity indicating that *P. woesei* is a sister strain or even the parent strain of *P. furiosus*. *P. woesei* may have acquired by lateral gene transfer more than 100 ORFs from other organisms living in the same thermophilic environment to produce the type strain *P. furiosus*.

Introduction

The shallow marine volcanic vents of Vulcano Island, Italy, have proven to be a rich source of thermophilic archaea. More than a dozen organisms have been isolated from this location (2, 21, 25, 26, 33, 49, 56, 57, 61, 62, 68) including two species of *Pyrococcus*, *P. furiosus* (25) and *P. woesei* (68). *P. furiosus* was the first of these organisms to be discovered in the Vulcano ecosystem and is now one of the best studied of the hyperthermophilic archaea. It grows optimally near 100 °C and utilizes peptides and carbohydrates as carbon and energy sources, generating organic acids and hydrogen or, if elemental sulfur is present, hydrogen sulfide as end products. The physiology of *P. woesei* appears to be very similar to that of *P. furiosus*. They have the same growth temperature range and use the same carbon sources and terminal electron acceptors (25, 68). The genome of *P. furiosus* has been sequenced. It is 1.9 Mb in size and contains over 2000 open reading frames (ORFs) (50). Although the genome of *P. woesei* has not been sequenced, nineteen protein sequences and two RNA sequences are available in the public database, all of which show at least 99% identity to their homologs in *P. furiosus* (4, 12-14, 16-20, 34, 40, 51, 63, 65, 70). This includes their 16S rRNA sequences, which are 100% identical (65).

The question arises as to how these two *Pyrococcus* species originated and the evolutionary relationship between them. This is an intriguing issue given the fact that they are found in the same geothermal environment. Indeed, because of the identity of their 16S rRNA sequences, the striking similarities in their physiological properties, and the disruption of a putative Na^+/H^+ antiporter gene (napA, PF0275) by an insertion

sequence, it was recently concluded that *P. woesei* should be classified as a subspecies of *P. furiosus* (65). To evaluate the overall genetic differences between the two organisms, we have utilized DNA microarrays based on the complete genome of *P. furiosus* (54, 55). The fundamental question to be addressed is, does *P. woesei* contain homologs of all the genes found in *P. furiosus*? Moreover, if the answer is no, what are the consequences of any differences, in terms of evolution, physiology, and metabolism? Genome-based DNA microarray comparisons have so far been restricted to mesophilic bacteria, where the goals were to determine the presence or absence of genes associated with pathogenic and non-pathogenic strains (9, 27, 32, 41, 48, 52, 67). We show here that the results of DNA microarray comparisons allow testable predications to be made about physiology and metabolism. In addition, the whole genome approach also provides an opportunity to gain insight into interactions between members of the Vulcano environment. This may provide a means to assess global genetic exchanges that potentially harken from a time when primitive archaeons lived on a hot earth and acquired or disseminated genetic innovation, such as stress resistance or utilization of a carbon source, for survival.

Materials and Methods

Array design and DNA preparation. Microarray slides were designed and processed as previously described (54, 55). *P. furiosus* (DSM 3638) and *P. woesei* (DSMZ 3773) were grown in 1 L culture bottles using maltose or peptides as the carbon source (64). Cells were harvested at the end of exponential growth and genomic DNA was isolated by a phenol/chloroform protocol (53).

Preparation of labeled DNA and hybridization conditions. Labeled DNA was prepared using the Prime-It Fluor kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions, except that a dUTP-aminoallyl tag (Sigma, St. Louis, MO) was used. Tagged DNA products and Alexa labeled DNA were purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA). Aminoallyl-labeled DNA was coupled with Alexa dyes 488, 546, 594 or 647 (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. Labeled genomic DNA samples were hybridized to the microarray slide containing PCR products to all 2,065 ORFs in the *P. furiosus* genome (54, 55) using a Genetac hybridization station (Genomic Solutions, Ann harbor, MI) for 14 hours at 65 °C. The slides were then washed for 20 sec each in 2X sodium chloride-sodium citrate buffer (SSC)/0.1% Tween-20, 0.2X SSC/0.1% Tween-20, 0.2X SSC, rinsed in distilled water and blown dry with compressed air. Fluorescence intensities of the four dyes, which represented one experiment in triplicate with one control, were measured using a Scan Array 5000 slide reader (Perkin Elmer, Boston, MA) with the appropriate laser and filter settings.

Data analysis. Spots were identified and quantitated using the Gleams software package (Nutec, Houston, TX). The relative fluorescent intensities were averaged from three sets of microarray data generated from slides that contained the *P. furiosus* genome printed in triplicate (9 arrays total). Spots giving negative fluorescent intensities with *P. woesei* DNA (78 of the 2065 spots examined) were converted to an arbitrary value of 200 units and those giving negative fluorescent intensities with *P. furiosus* DNA (16 of 2065) were converted to the minimum detection limit of 2000 units. Instead of eliminating negative *P. furiosus* numbers, the conversions were used to keep positive *P. woesei* hybridizations to the spot even though the *P. furiosus* control did not. The fluorescent intensities collected from the *P. woesei* data set were divided into the *P. furiosus* data set of fluorescent intensities. The resulting values were then multiplied by -1 and added to the number 100. Values less than 98 were taken to indicate that a given *P. furiosus* gene did not have a homolog in the *P. woesei* genome (corresponding to where *P. woesei* fluorescent intensity was 50% of *P. furiosus* fluorescent intensities). ORF analysis was conducted using the InterProScan tool (v.3.3; http://www.ebi.ac.uk/interpro/). Insertion sequences (IS) were analyzed using ISfinder (http://www-is.biotoul.fr/). Homologs of *P. furiosus* ORFs in the genomes of *P. abyssi* (8, 23), *P. horikoshii (30, 39)* and *T. kodakaraensis* are defined as those ORFs encoding proteins having at least 75% sequence similarity over at least 75% of the protein length when analyzed by BLASTP.

PCR and Sequencing. Primers were synthesized and PCR products were sequenced by the University of Georgia Integrated Biotech Laboratories (http://www.ors.uga.edu/ibl/index.html/). PCR analyses were carried out in triplicate with different stringency annealing temperatures and positive controls. Primers used to amplify the Mal I locus were: forward 5' –AAT ACG CTC ATA GAA TCA AAG- 3', and reverse 5' –CCC TAT GAC TGC CTT TGG ATT- 3'. All PCR reagents were obtained from Stratagene, and standard molecular biology techniques were as described (53).

Growth Studies. The two types of maltose used in the growth studies were 95% grade (M2250, Sigma) and 99% grade (M9171, Sigma). Roussin's Black Salt (RBS, $Na^+[Fe_4S_3(NO)_7]^-$) (7) was provided by Professor Martin Hughes (King's College, London). The dried powder was suspended in degassed water to a final concentration of approximately 0.9 μM prior to use.

Pulse field Gel Electrophoresis (PFGE). The procedure was adapted from that described by Robb *et al.* (5). Gel plugs were made by suspending cells of *P. furiosus* or *P. woesei* to a concentration of 5 x 10^9 cells/mL in 1% (w/v) agarose. The plugs were incubated with 0.1 M EDTA, 1% (w/v) cetyl trimethylammonium bromide, 1% (w/v) SDS, 1% (v/v) Triton X-200, and proteinase-K (2.0 mg/ml) for 24 hrs at 42 °C. Plugs were washed twice with 10 mM Tris buffer, pH 8.0, containing 1 mM EDTA (TE buffer) for 15 min at 4 °C, incubated with 1 mM phenylmethylsulfonylfluoride (PMSF) in 10 mM TE buffer at 23°C for 2 hr, washed with 10 mM TE buffer at 23°C for 2 hr, and were

equilibrated with 1X restriction enzyme (RE) buffer (New England Biolabs, Ipwich, MA) for 20 min at 4 °C. Using fresh 1X RE buffer, each plug was incubated with 25 U of Not I (New England Biolabs) for 16 hrs at 37°C. Plugs were inserted into a 1% (w/v) agarose gel and electrophoresed using the CHEF-DR II system (Bio-Rad, Hercules, CA.) for 20 hrs at 200 V with alternating field (90°/90s for 1 hr and 90° from 1 to 25 s over 19 hr). DNA bands were stained with ethidium bromide.

Results

Genomic DNA (gDNA) from *P. woesei* was hybridized to the DNA microarray containing spots representing the 2,065 ORFs annotated in the *P. furiosus* genome. The fluorescent intensities were compared directly with those measured using gDNA from *P. furiosus*. The results showed that close homologs of 1890 ORFs (92%) of the 2,065 *P. furiosus* ORFs were present in the *P. woesei* genome. This included all twenty-one sequences of *P. woesei* genes available in the NCBI database (http://www.ncbi.nlm.nih.gov/) that are known to be (virtually) identical by direct sequence comparisons. The remaining 175 *P. furiosus* ORFs (representing 8% of *P. furiosus* genome) were not detected in *P. woesei* DNA at a significant level by microarray analysis, implying that close homologs of those genes are missing from the *P. woesei* genome. It is possible that the apparent absence of some genes is because they are highly divergent. However, this would seem unlikely given the almost complete identity of all genes (and proteins) so far examined in the two species. The arrangement of the proposed missing ORFs on the *P. furiosus* genome is shown in Figure 2.1. As is readily apparent, a striking feature is that in *P. furiosus* these missing ORFs form clusters or ORF islands. This pattern is not unlike what was previously proposed (24) suggesting that these ORFs form such islands or gene cassettes because of functional interactions. If *P. woesei* is as closely related as suspected and synteny is conserved then the regions of missing ORFs are restricted to specific areas of the genome.

The veracity of the microarray results was assessed by direct PCR analysis using primer pairs covering 137 ORFs out of the 175 ORFs proposed by the microarray results (78% coverage). Of the 137 ORFs analyzed, PCR products of the expected size were obtained for all of them using gDNA from *P. furiosus*, but only 32 yielded PCR products using *P. woesei* gDNA. This analysis therefore confirmed the absence in the *P. woesei* genome of homologs of 105 *P. furiosus* ORFs (or 77% of those indicated by the DNA microarray). The list of *P. furiosus* ORFs missing from the *P. woesei* genome is given in Table 2.1. Of the 105 ORFs, most of them (93 ORFs) were present in 20 gene clusters consisting of two or more genes, and these are indicated in Table 2.1 (where shaded boxes indicate potential operons). Analysis by InterPro of the amino acid sequences revealed that 37 of the 105 ORFs (35%) are proteins of unknown function with no known homolog in other organisms. Conversely, of the 444 ORFs that are annotated as being hypothetical in *P. furiosus*, 407 of them appear to be present in the *P. woesei* genome according to the DNA microarray analysis. This would suggest that these hypothetical ORFs in *P. furiosus* do indeed encode proteins. The results of a BLASTP analysis of the 105 *P. furiosus* ORFs (not present in *P. woesei*) to three closely-related species *P. abyssi* (8, 23), *P. horikoshii* (30, 39) and *T. kodakaraensis* (28) is shown in Table 2.1. Only seven of the *P. furiosus* ORFs have homologs in the genomes of all three species and only eleven ORFs had homologs in two of the three organisms. In *P. furiosus,* these ORFs are scattered about the genome, except for the gene cluster PF0764-PF0770, a homolog of which is found in *P. abyssi* but not in the other two organisms.

To gain further insight into the differences between the genomes of *P. furiosus* and *P. woesei*, and the proposed absence of 105 ORFs in the latter, a PFGE analysis was performed on DNA isolated from both species after digestion using the Not I restriction enzyme. For *P. furiosus* DNA, this enzyme should generate six DNA fragments of approximately 43, 132, 224, 385, 416 and 709 kbp. Assuming that the two genomes differ only by these 105 ORFs, treatment of *P. woesei* DNA should also yield six fragments, two of which (42 and 132 kbp) are the same as those in *P. furiosus*. Each of the other four fragments from *P. woesei* are predicted to be smaller (206, 371, 401 and 667 kbp) than the corresponding fragments from *P. furiosus* DNA. PFGE analysis revealed the expected six bands from *P. furiosus* DNA, and six bands were also seen after digestion of *P. woesei* DNA, all of which corresponded to the *P. furiosus* DNA fragments (data not shown). Five of the bands appeared to be the same in both species since differences of less than 20 kb were not resolved. However, the sixth fragment (667 kb) from *P. woesei* DNA was distinguishable from that of the *P. furiosus* DNA (which was predicted to be 41 kb larger). Given that no fragments were observed from *P. woesei* DNA that were larger than predicted, it is concluded that this genome is approximately the same size as that of *P. furiosus* and lacks all of the 105 ORFs (equivalent to 88.8 kbp) predicted by the microarray and PCR analysis.

The power of the comparative DNA microarray approach is that it enables predictions regarding metabolism and physiology. Thus, of the ORFs listed in Table 2.1, of particular interest are those encoding proteins of known or predicted functions that are amenable to phenotypic analysis. One such gene cluster is PF1737-PF1751, which

includes the Mal I operon found in *P. furiosus* (1, 54) and in the related genus *Thermococcus* (49, 66). This operon encodes an ABC type maltose/trehalose transporter (malEFG and malK, represented by PF1739-PF1741, and PF1744 respectively) as well as a trehalose-degrading enzyme (PF1742) (1, 31, 35, 36, 43, 66). Interestingly, *T. litoralis*, which contains the Mal I operon, was also isolated from a shallow marine volcanic vent at Vulcano Island, Italy. In contrast, as is evident from the BLASTP results (Table 2.1), *P. horikoshii*, *P. abyssi* and *T. kodakaraensis* do not have a complete Mal I gene cluster. These three organisms were isolated from deep sea hydrothermal environments (3, 23, 30), implying perhaps that the availability of the Mal I gene cluster is limited to the vicinity of Vulcano island. However, the apparent absence of the Mal I operon from *P. woesei* is inconsistent with the report that the organism is able to grow on maltose (68). Indeed, in our hands *P. woesei* exhibited very good growth (densities $>10^8$ cells/mL) using the standard *P. furiosus* maltose-containing medium (64). This discrepancy was resolved by the finding that *P. woesei* did not exhibit significant growth on high purity (99%) maltose, rather than the technical grade (95%, which contain 5% glucose and polysaccharides) usually used (Figure 2.2). Hence, in agreement with the DNA microarray analysis, maltose does not support growth of *P. woesei*.

A second gene cluster of interest in *P. furiosus,* but absent in *P. woesei,* is PF0691-PF0695. This contains an ORF (PF0694) encoding a protein that has between 32 and 65% sequence similarity to the flavoprotein nitric oxide reductase (NOR) from the anaerobic bacteria, *Moorella thermoacetica* (58), *Desulfovibrio vulgaris* (59) and *D. gigas* (58, 60). The protein encoded by PF0694 has the conserved residues required to

coordinate the binuclear non-heme iron site found in NOR (10, 15, 29). Analysis of the genome sequences available for 23 archaea revealed that only six of them have a homolog of the bacterial NOR. These include *Archaeoglobus fulgidus* and the methanogens *Methanobacterium thermoautotrophicum, Methanococcus janaschii, Methanosarcina (Ms.) acetivorans,* and *Ms. mazei.* As indicated in Table 2.1, a close homolog of PF0694, which is annotated by InterPro as NO synthase, is not present in *P. abyssi, P. horikoshii* or *T. kodakaraensis*.

In light of the putative NOR system in *P. furiosus*, and its apparent absence in *P. woesei*, the question arose as to whether the two organisms exhibited any differences in their response to reactive nitrogen species (RNS). However, the sensitivity of archaea to RNS has not been reported. To investigate the responses of the two *Pyrococcus* species, we used the NO-generator known as Rousssin's Black Salt (RBS). This iron-sulfur-nitrosyl compound delivers seven molar equivalents of NO, and is a potent antimicrobial agent (6, 7, 38, 46, 47). If *P. woesei* does not contain a homolog to PF0694, the organism should be more susceptible to the NO-generator than *P. furiosus*. As shown in Figure 2.3, growth studies using RBS showed that *P. furiosus* is not significantly affected by the addition of 0.9 μM RBS while under the same conditions, the *P. woesei* cultures were no longer viable. These results strongly suggest that the cluster PF0691-PF0695, and particularly PF0694, play a key role in detoxifying RNS (37).

It is therefore clear that *P. woesei* and *P. furiosus* share a close genetic origin. Interestingly, analysis of the *P. furiosus* genome revealed that the ORF islands not

detected in *P. woesei* are either bracketed by, or are found in close proximity to, insertion sequences (IS). This is illustrated in Figure 1 by the bar coding. A notable exception is the putative gene cluster (PF0691-PF0695) that contains NOR (Table 2.1). However, this has an IS on one side (PF0756) and on the other, adjacent to PF0688, there is a ~3.5 kb stretch of long cluster of tandem repeats (LCTR). The tandem repeat that composes the LCTR is 29 nucleotides long. An LCTR is also located next to PF0025-PF0032, another ORF cluster absent in *P. woesei* (see Table 2.1). LCTRs are non-coding repeat sequences in tandem believed to behave like mobile elements and that have been proposed to participate in gene transfer (69). The position of LCTRs next to some of the ORF clusters that are not present in *P. woesei* strongly supports such a proposition.

ISs are mobile elements that can transpose within the genome or into extra chromosomal elements (11, 45). The Mal I gene cluster found in *P. furiosus* (but not in *P. woesei*) is particularly noteworthy as it is packaged as a composite transposon. In other words, the Mal I is bracketed by two identical ISs, and the whole composite transposon (including ISs) is flanked by matching direct repeats (DR), an indication of insertion as a complete composite transposon. In fact, the sequence of the Mal I gene cluster is virtually identical in *P. furiosus* and *T. litoralis* and it was proposed that a lateral gene transfer event was responsible (22, 36). To investigate whether the nature of IS and DR elements around the Mal I gene cluster could provide insights into the phylogenetic relationship between *P. furiosus* and *P. woesei,* the sequence of the relevant region in *P. woesei* was determined. PCR primers were designed to anneal outside of the vicinity of the composite Mal I transposon in *P. furiosus* to determine what was present

in the corresponding region in *P. woesei*. The same experiment with *P. furiosus* produced a PCR product that was approximately 17 kb (the Mal I composite transposon is 17854 bases in length) while *P. woesei* produced a fragment that was about 780 bp (see Figure 2.4). The sequence of the PCR product from *P. woesei* (accession number DQ202294) revealed that synteny of surrounding ORFs was conserved between *P. woesei* and *P. furiosus*. An 8-nucleotide sequence, CAGGAGGA, was found in the *P. woesei* locus where in *P. furiosus* the Mal I is located. This sequence is not spurious, as the DRs that bracket the *P. furious* Mal I composite transposon have the same sequence (Figure 2.5).

Discussion

Insertion sequences are suspected of playing key roles in shaping the *P. furiosus* genome leading to evolutionary divergence from *P. abyssi* and *P. horikoshii* (42, 44, 69). A total of twenty eight transposases are annotated in the *P. furiosus* genome and our analysis of them shows that they comprise four groups. They have been given the formal names of ISPfu1 (8 isoforms, family IS6), ISPfu2 (11 isoforms, family IS6), ISPfu3 (5 isoforms, family IS982) and ISPfu5 (4 isoforms, family IS6) after analysis by ISFinder (http://www-is.biotoul.fr/). The ISs that bracket the Mal I gene cluster are isoforms from the group ISPfu1. This is a member of the IS6 family, which transpose via replicative transposition. This is accomplished by formation of cointegrates between the donor and target sites that resolve leaving a copy of the IS in the target and the donor site (45). Once a member of the IS6 family inserts into a locus, that copy will remain at the new location, potentially replicating to other target sites in time. However, the PF1735/PF1753 locus of *P. woesei* does not contain an IS, or a composite transposon, nor any indication that the locus has been involved in replicative transposition. Assuming that the archaeal ISs follow the observed IS6 replicative progression, we conclude that *P. woesei* is a sister strain and possibly the parent strain of *P. furiosus*. *P. furiosus* would therefore be the type strain, and presumably acquired ORFs (missing from *P. woesei*) from external sources at temperatures near 100°C in the same hydrothermal environment.

ISs likely play a pivotal role in shaping the genomes of the thermophilic community found at Vulcano Island and at similar locations on Earth. The use of DNA microarrays enables the first step to be taken towards understanding genomic phenomena

such as the dissemination of gene cassettes within a community. This places the focus of attention on gene clusters found within archaeal communities rather than individual genes or the individual organism. However, as demonstrated herein, additional molecular and phenotypic characterizations are necessary to confirm the implications of array results.

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Table 2.1. *P. furiosus* ORFs that lack homologs in the *P. woesei* genome based on DNA microarray and PCR analyses[a]

ORF	A	B	C	InterPRO Annotation	ORF Number	A	B	C	InterPRO Annotation
PF0025	*	*	*	Glutamine amidotransferase, II	PF0735				No InterPRO entry
PF0026	*	*	*	DNA polymerase, beta-region	PF0737				No InterPRO entry
PF0029				No InterPRO entry	PF0738				SAM binding protein
PF0030				No InterPRO entry	PF0739				Bacterial regulator, AsnC/Lrp
PF0031				PLP-enzyme, beta subunit	PF0740			*	Copper-transporting ATPase
PF0032				No InterPRO entry	PF0742			*	Ferritin and Dps
PF0034		*		No InterPRO entry	PF0743				CoA-binding domain
PF0035		*		ABC transporter	PF0744			*	ABC transporter
PF0037				ARM repeat fold	PF0758	*	*		HEPN, nucleotide-binding
PF0038	*	*		Beta-lactamase-like	PF0760	*		*	Transposase, IS605 OrfB
PF0041	*	*	*	Elongator protein 3/MiaB/NifB	PF0762	*	*		No InterPRO entry
PF0151	*			Glycoside hydrolase, family 5	PF0763				No InterPRO entry
PF0152		*	*	Protein of unknown function	PF0764	*	*	*	Archaeal ATPase
PF0154	*	*	*	Glutamine amidotransferase, II	PF0765		*		6-phosphogluconate DH, C-term
PF0365				Protein of unknown function	PF0766		*		Oxidoreductase, N-terminal
PF0366	*	*	*	No InterPRO entry	PF0767		*		DegT aminotransferase
PF0685				Protein of unknown function	PF0768		*		Transferase hexapeptide repeat
PF0687				Transcription factor TFIIB	PF0769		*		Glycosyl transferase, family 2
PF0689				Prismane-like	PF0770		*		UTP-glucose-1-Pi transferase
PF0691		*		Lambda repressor-like	PF0772	*			DNA polymerase, beta-like
PF0692		*		Prismane	PF0773				No InterPRO entry
PF0693				SirA-like	PF0775				No InterPRO entry
PF0694				Flavodoxin/NO synthase	PF0776			*	No InterPRO entry
PF0695		*		Protein of unknown function	PF0777				No InterPRO entry
PF0697				Sugar transporter superfamily	PF0781		*	*	Nucleotide binding protein, PINc
PF0698				No InterPRO entry	PF0783		*		No InterPRO entry
PF0701	*	*		No InterPRO entry	PF0790				No InterPRO entry
PF0702	*	*		Peptidase M24	PF0791				Glycosyl transferase, group 1
PF0703				No InterPRO entry	PF0792				No InterPRO entry
PF0704				Protein of unknown function	PF0794				UDP-N-Ac gluc.-2-epimerase[b]
PF0705				Cytochrome c biogenesis	PF0795				Glycosyl transferase, group 1
PF0706				Protein of unknown function	PF0796				Transferase hexapeptide repeat
PF0707				4-oxalocrotonate tautomerase	PF0797				No InterPRO entry

PF0708				Extrusion protein MatE	PF0798				Glycosyl transferase, family 2
PF0712				No InterPRO entry	PF1337	*	*		TENA/THI-4 protein
PF0713		*		Winged helix DNA-binding	PF1339				Protein of unknown function
PF0715				Nitroreductase	PF1340				Protein of unknown function
PF0716				6-phosphogluconate DH	PF1737				No InterPRO entry
PF0717				SAM binding motif	PF1738				Ribokinase
PF0718				No InterPRO entry	PF1739				Solute-binding protein, family 1
PF0719				No InterPRO entry	PF1740				Membrane transport component
PF0720				No InterPRO entry	PF1741				Membrane transport component
PF0721				NADPH: FMN reductase	PF1742	*			Glycosyl transferase, group 1
PF0722				Alkyl hydroperoxide reductase	PF1743	*			Protein of unknown function
PF0723				Iron permease FTR1	PF1744	*	*	*	ABC transporter
PF0725				CoA-binding domain	PF1745	*	*		L-fucose isomerase
PF0727				No InterPRO entry	PF1746				Glycoside transferase
PF0729				4Fe-4S ferredoxin	PF1747				Protein of unknown function
PF0730			*	No InterPRO entry	PF1748			*	Membrane transport component
PF0731				Ferritin-like	PF1749			*	Membrane transport component
PF0732				ABC transporter	PF1750	*	*	*	ABC transporter
PF0733				ABC transporter	PF1751	*	*		Thiamine ABC transporter
PF0734				No InterPRO entry					

[a]The shaded blocks represent potential ORF clusters (where adjacent ORFs can be on the positive or negative strand). Asterisks indicate the presence of a homolog in A, *P. horikoshii*; B, *P. abyssi*; or C, *T. kodakaraensis.* See text for details.

[b]UDP-N-Acetylglucosamine-2-epimerase

Figure 2.1: Hybridization of genomic DNA from *P. woesei* to the *P. furiosus* DNA microarray. The data were normalized as described in the Methods. The abscissa is numbered by *P. furiosus* ORF 1 to 2065. The bar graph at the top of figure denotes positions of *P. furiosus* insertion sequences.

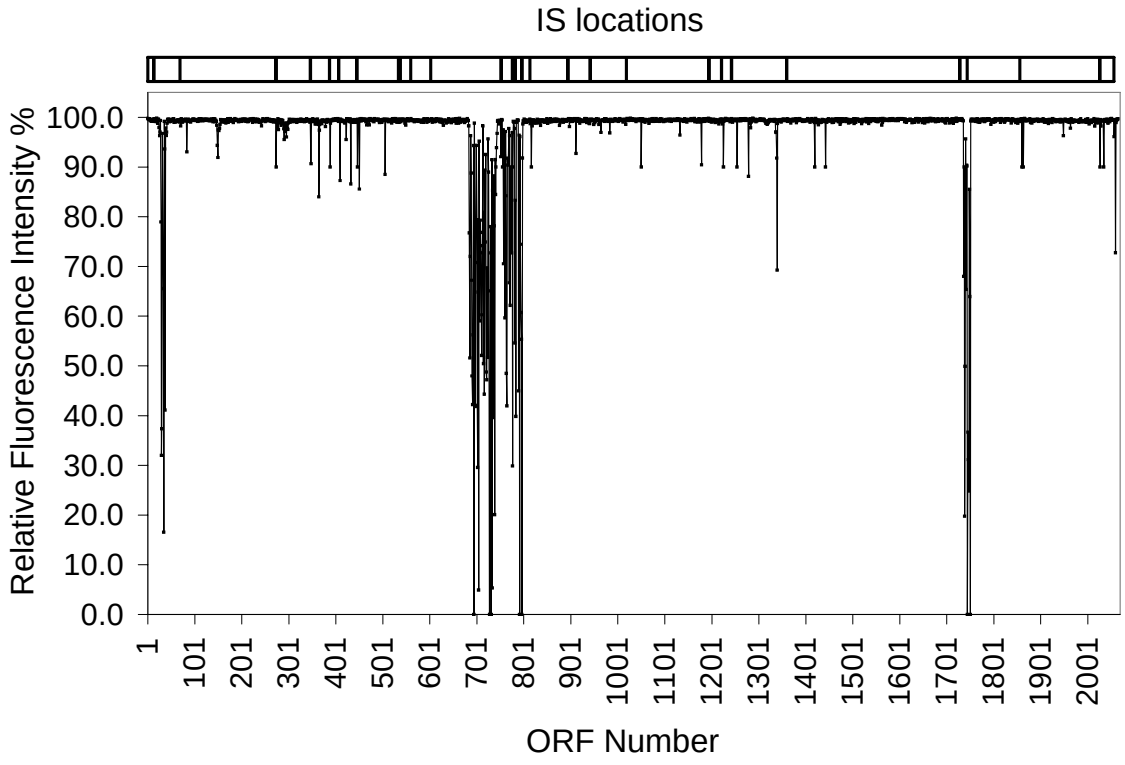


Figure 2.2: Growth of *Pyrococcus* species on 99% pure maltose. The curves represent *P. furiosus* (♦) and *P. woesei* (●).

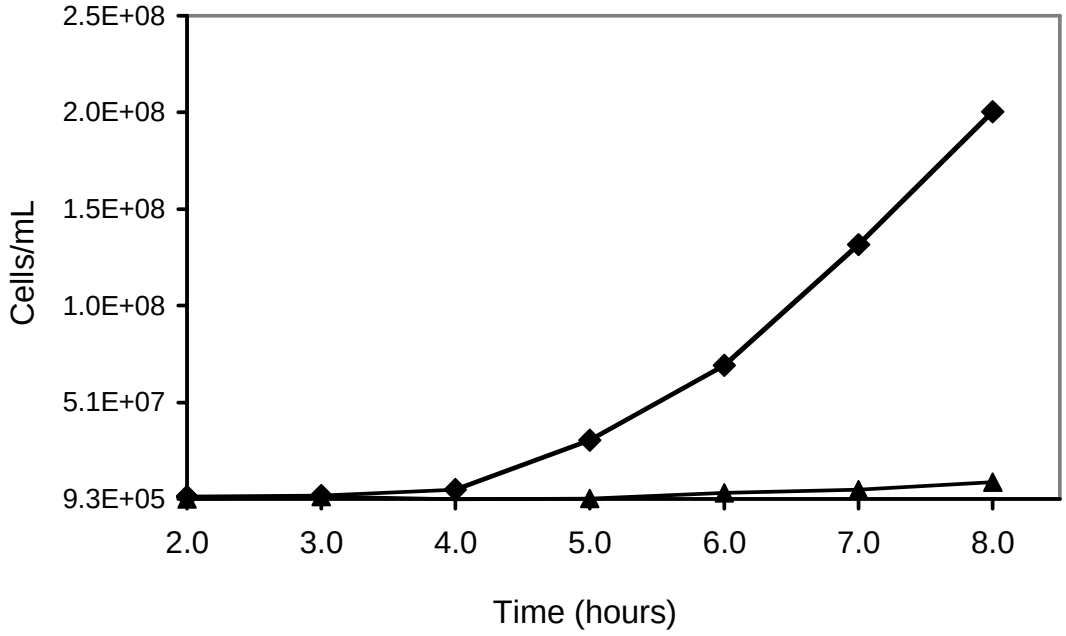


Figure 2.3. Growth of *P. woesei* and *P. furiosus* in the presence of the NO-generator RBS. Arrows indicate points of addition of RBS (0.9 μM). The symbols represent *P. furiosus* with (▲) and without (♦) RBS, and *P. woesei* with (■) and without (●) RBS.

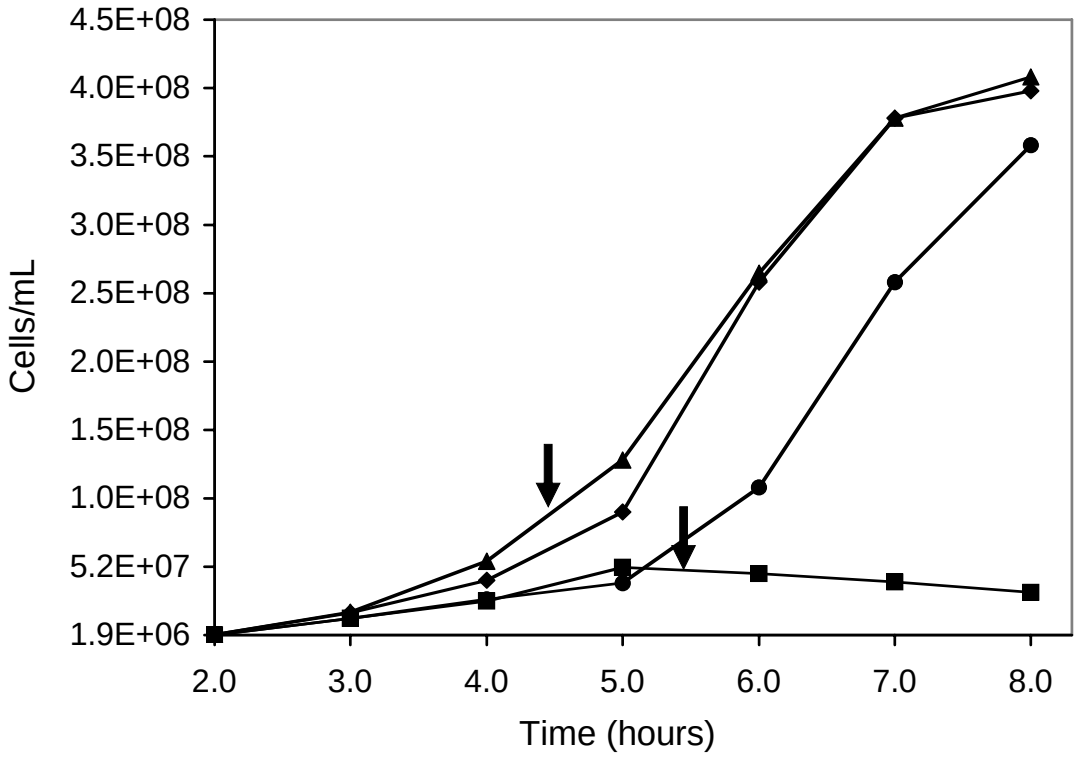


Figure 2.4. Products of the Mal I region of *P. furiosus* and *P. woesei* by PCR analysis. The products were analyzed on an agarose gel (0.5% w/v) and stained with ethidium bromide. The DNA ladder Lambda/Hind III – Phi X174/Hae III was from Strategene.

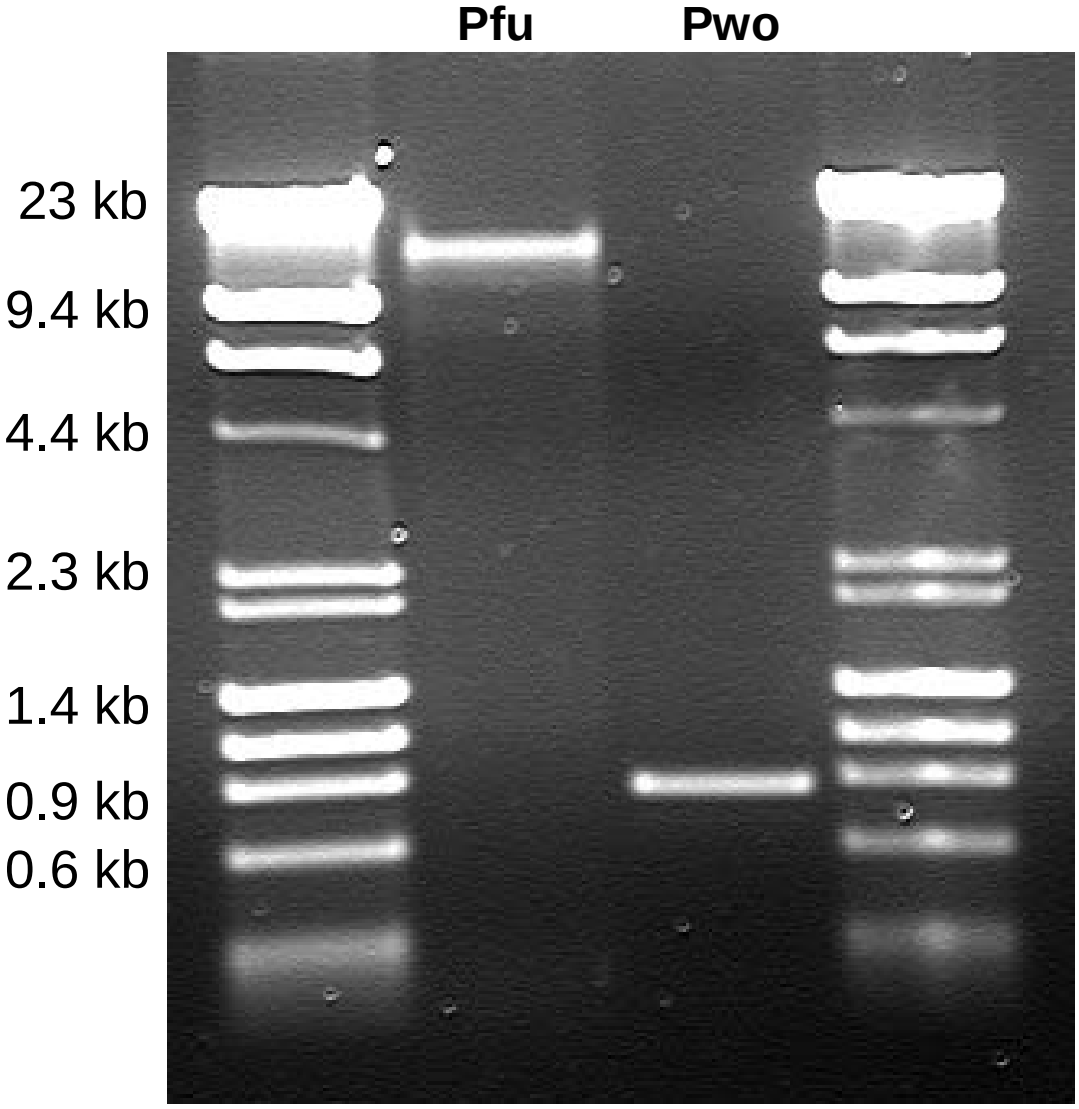
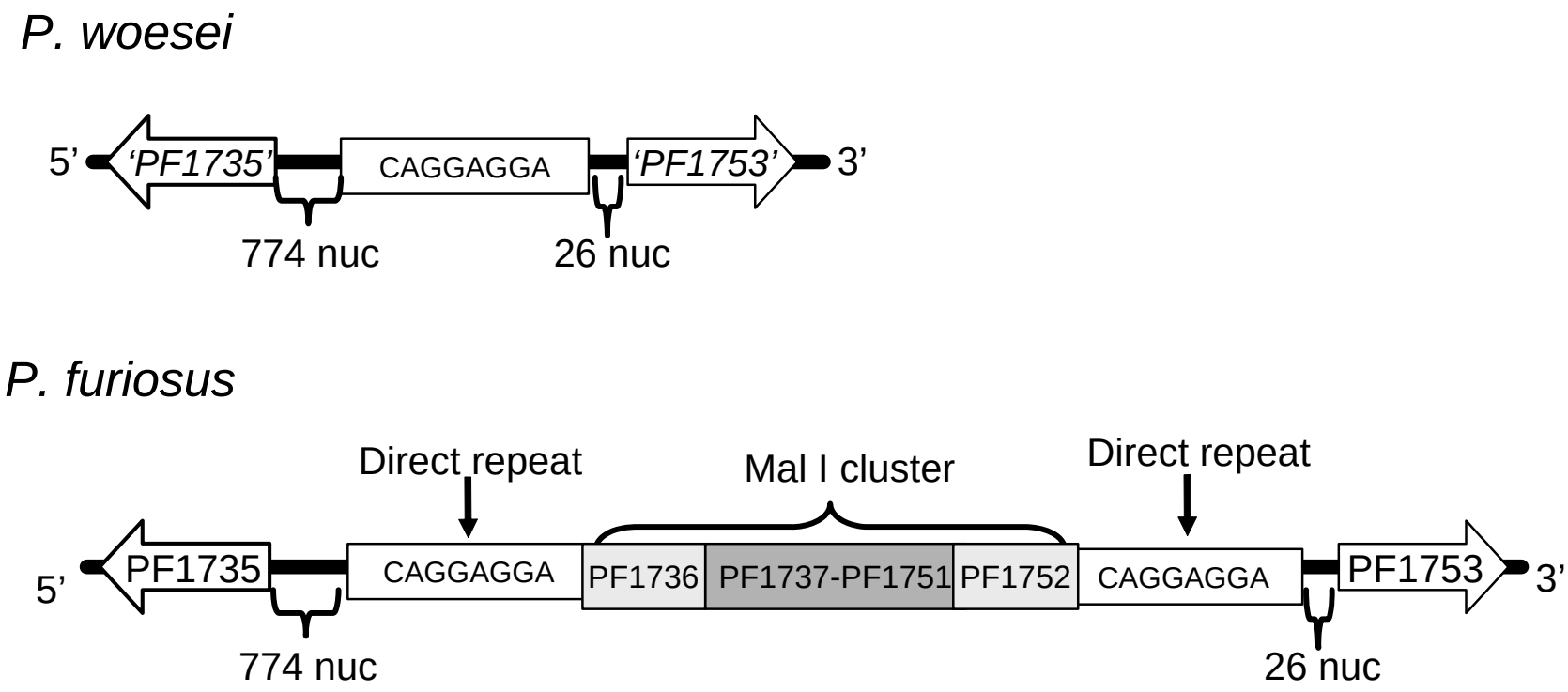


Figure 2.5. Representations of the sequences of the PCR products of the Mal I regions of *P. furiosus* and *P. woesei*. See text for details. The sequenced ORFs from the unsequenced *P. woesei* genome have been labeled '*PF1735*' and '*PF1753*'. These two ORFs in *P. woesei* are identical in nucleotide sequence to the corresponding *P. furiosus* ORF.



CHAPTER 3

CHARACTERIZATION OF THE ANTIMICROBIAL EFFECTS OF ROUSSIN'S BLACK SALT ON THE HYPERTHERMOPHILIC ARCHAEON *PYROCOCCUS FURIOSUS*

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Abstract

Roussin's Black Salt (RBS) is a broad spectrum antimicrobial agent that has proven very effective in controlling pathogenic anaerobes such as *Clostridium* sp. both in the vegetative and spore state. RBS consists of a cubane iron-sulfur cluster with seven moles of nitric oxide ($[Fe_4S_3(NO)_7]^-Na^+$). It has been generally assumed that the release of nitric oxide is the cause of the cytotoxic effects of RBS although the mechanism is unknown. Using the archaeon *Pyrococcus furiosus* it is demonstrated with growth studies, membrane analyses, and scanning electron microscopy that nitric oxide does not play a role rather, the mechanism of RBS toxicity involves membrane disruption. It is proposed that RBS represents a new class of molecule, an inorganic surfactant, with antimicrobial activity. Moreover, insoluble elemental sulfur (S°), which is reduced by *P. furiosus* to hydrogen sulfide, prevents membrane disruption by RBS. It is proposed that S° also directly interacts with the membranes of *P. furiosus* during its transfer into the cell ultimately for reduction by a cytosolic NADPH sulfur reductase.

Introduction

Historically, reactive nitrogen species (RNS) such as nitrite have been used effectively to prevent bacterial spoilage of fish and meat (1, 2). This toxicity is thought to be the result of the generation of nitric oxide (NO) which then chemically modifies key enzymes and proteins via primary thiol groups or iron-sulfur clusters (1, 3-8). However, the true mechanism by which RNS inhibit microbial growth is poorly understood (4, 9-11). Health concerns over possible connections between nitrite and cancer has caused a steady decline in the amount of nitrite used in American and European foods (12, 13). A more complete understanding of the action of RNS and related molecules is clearly necessary.

Herein we focus on a type of RNS termed Roussin's Black Salt (RBS). RBS is even more bacteriocidal than nitrite (2, 14) and was discovered in 1858 by the French scientist M. L. Roussin, who synthesized it by heating nitrite, iron and sulfide (14). It is a broad spectrum antimicrobial agent that inhibits the growth of Gram-positive and Gram-negative prokaryotes, including many pathogenic strains of anaerobes including species of *Clostridia* (1, 11, 15-17). Remarkably, RBS is toxic to spores as well as vegetative cells (18). As indicated by the formula, $Fe_4S_3(NO)_7Na$, RBS has a cubane structure with seven moles of nitric oxide positioned around an iron-sulfur core (19). The presence of NO groups understandably leads to the assumption that the toxicity of RBS is due to the release of NO (2, 8, 11, 20). Indeed, over many days RBS slowly decomposes aerobically and does release NO (6, 21-23), but under anaerobic conditions the release of

NO is not observed (6). In contrast to most antibiotics, RBS is stable up to 120°C (2, 14) and therefore is of potential utility in developing genetic systems in microorganisms that thrive at high temperatures, and particularly those that grow anaerobically. In addition, in contrast to several bacterial antibiotics, RBS might also be effective against members of the archaea.

Pyrococcus furiosus is a non-pathogenic anaerobe. It has been studied extensively as a hyperthermophile and as an archaeon (24-26), making it an excellent model organism with which to investigate the mechanism of RBS. *P. furiosus* grows optimally near 100°C and utilizes carbohydrates as carbon sources generating organic acids and CO_2 as end products. Protons or elemental sulfur (S^o) can be used as terminal electron acceptors producing hydrogen or hydrogen sulfide, respectively, although S° is the preferred electron sink (27). Herein it is shown that the growth of *P. furiosus* is very sensitive to RBS but that its mechanism of action does not involve NO. Moreover, the effect of RBS also provides insight into how the organism likely metabolizes S°.

Materials and Methods

Roussin's Black Salt. RBS was provided by Dr. Martin Hughes (Kings College, London, United Kingdom). Stock solutions of 1.8 mM RBS were prepared anaerobically in glass-distilled water or in the *P. furiosus* growth medium. Concentrations of RBS were estimated using an extinction coefficient at 275 nm of 29,650 (1/cm.M) (21, 23). The stability of RBS was determined at 95°C using an anaerobic sample of RBS (60 μM in *P. furiosus* growth medium at pH 6.8). For the S-nitrosothiol assays, RBS, nitric oxide and nitrite (each 1 mM) were prepared in 10 mM 4-(2-hydroxyethyl)piperazine-1-propanesulfonic acid (EPPS) buffer, pH 7.0. Under anaerobic conditions at 85°C, each was added to an equal volume of various thiol compounds, also at a concentration of 1 mM. Formation of the S-nitrosothiol was measured by visible spectroscopy at 540 nm (4, 7, 28).

Growth Studies. *P. furiosus* (DSM 3638) was cultured as described previously (29) using 0.5% (w/v) maltose (95% grade, Sigma, St. Louis, MO) as the carbon source with and without S° (0.1%, w/v). Cell density was measured by direct counting using a BX41 Olympus phase-contrast microscope (Olympus, Center Valley, PA) and a Hausser Scientific Partnership counting chamber (Horsham, PA). The toxicity of *P. furiosus* to RBS was determined by growing the organism to cell densities of ~5.0 x 10^7 cells/mL and injecting RBS to final concentrations of 0.5 - 5.0 μM. Elemental sulfur (0.1%, w/v), where indicated, was added up to 30 min before the addition of RBS into the culture. Cold shift assays were conducted by transferring cultures (5.0 x 10^7 cells/mL) grown at

98ºC to 4ºC for 1 hour. RBS (2 μM) was added and the cultures were monitored by cell counts.

Membrane Preparations. Membranes were prepared under anaerobic conditions from cells (400 mL cultures) harvested when the cell density reached 5.0 x 10^7 cells/mL after treatment with RBS (20 μM). Membranes were collected by centrifugation at 113,000 x *g* for 2 hours (Optima L-90K, Beckman-Coulter, Ramsey, MN). The membrane pellet was washed twice with degassed glass-distilled water and resuspended in 2.5 mL degassed glass distilled water. Samples (20 - 1200 μL) were then injected into *P. furiosus* cultures (10 ml) at 98°C and cell densities were determined after 30 min. Membrane pellets containing RBS (if pellets are assumed to have absorbed the full concentration of 20 μM RBS, the 2.5 ml 'membrane' preparation will contain ~3.2 mM RBS) were also treated separately with colloidal sulfur (~4 mM) and polysulfide (4 mM) at 85ºC for 1 hour and were then resuspended in 2.5 mL degassed glass distilled water or 10 mM N-Cyclohexyl-2-aminoethanesulfonic acid (CHES) pH 10 buffer (to prevent the formation of elemental sulfur) for elemental sulfur and polysulfide, respectively. Polysulfide was prepared anaerobically by reacting 100 mM sodium sulfide with an excess of elemental sulfur ~6% (w/v) for 24 hour at 25°C. The toxicity of sulfur-treated RBS was assessed by adding the membrane preparation (20 – 1200 μl) to *P. furiosus* cultures (10 mL) at 25ºC for 30 min. Membrane experiments were repeated using nitrite (800 μM) or NO (40 μM) in place of RBS.

Scanning Electron Microscopy (SEM) preparation. RBS (1.0 μM) was injected into a culture of *P. furiosus* growing at 98°C (cell density of ~ 5.0 x 10^7 cells/mL) and samples (1 mL) were taken after 30 and 60 seconds. These were cooled to 4°C, fixed with 2% glutaraldehyde, washed three times with phosphate buffered saline (PBS), and fixed with 1% osmium tetraoxide (OsO_4) for 1 hour. Samples were dehydrated sequentially using 25, 50, 75, 85, 90, and 100% ethanol with three washes at each ethanol concentration, gently filtered through a 0.2 micron Millipore membrane using a Swinney filter (Millipore, MA), critical point dried, mounted on a post, and coated with ~153 Å of gold with a sputter coater (Structure Probe Inc, PA). The coated sample was scanned using a Leo 982 Field emission scanning electron microscope (Zeiss, MA) at the Center for Ultrastructural Research (University of Georgia, Athens, GA).

Cellular characterization: Fluorescent reporters, SYTO 9 and propidium iodide were used in the LIVE/DEAD BacLight Bacterial Viability kit (Molecular Probes, Eugene, OR). Samples (1.0 mL) of a *P. furiosus* culture grown with and without S° (0.1%, w/v) were centrifuged at 16,000 x *g* for 5 min, and the cells were resuspended in *P. furiosus* medium (3 ml) and collected by centrifugation (16,000 x *g* for 5 min). Cells were resuspended in growth medium (2 mL). The two fluorescent dyes (3 μL each) were added and the mixture was incubated at 25°C for 15 minutes in the dark. Fluorescence was measured with and without RBS (4 μM) using a Shimadzu RF-5301 PC spectrofluorophotometer by excitation at 480 nm and emission at 500 nm.

Results

RBS was toxic to a culture of *P. furiosus* in mid-exponential growth (~5 x 10^7/ml) at 98°C. As shown in Figure 3.1, the addition of RBS (2.0 μM) resulted in complete cell lysis as determined by light microscopy. Concentrations of RBS greater than 0.5 μM but less than 2 μM inhibited cell growth for up to 3 hours, after which time the culture resumed growth.

In order to investigate whether it was RBS or a product of its degradation that was responsible for the toxicity, the stability of RBS was determined under the growth conditions of *P. furiosus*. There was no detectable decomposition of RBS as measured by UV/visible spectroscopy when a sample (60 μM) was incubated anaerobically at 98ºC for 20 hours in the *P. furiosus* growth medium (without S°) (data not shown). Moreover, this heat-treated sample of RBS was as toxic to *P. furiosus* as an untreated sample (data not shown). RBS is therefore stable at high temperature and can be used as an antimicrobial agent under such conditions. It was assumed that the toxic effects of RBS were mediated by NO, which readily reacts with primary thiols groups to generate the S-nitrosothiol derivatives. To investigate the reactivity of RBS, samples of RBS, NO, or nitrite (each 1 mM) were prepared anaerobically in 10 mM EPPS buffer, pH 7.0, and these were incubated at 85°C for 30 minutes with L-cysteine, dithiothreitol, glutathione, 1-thioglycerol, thioglycolate, coenzyme A, or 2-mercaptoethanol. In contrast to NO and nitrite, which reacted with all thiols tested (data not shown), RBS did not generate a detectable S-nitrosothiol adduct with any of the primary thiols tested. These results indicated that RBS does not readily generate NO.

P furiosus can use insoluble S° as an electron acceptor, forming H_2S instead of reducing protons to H_2 (24). Surprisingly, *P. furiosus* cells in mid-exponential growth were not lysed by the addition of 2.0 μM RBS if S° was present, although they were if S° was absent (Figure 3.2A). A concentration of 20 μM was required to cause the degree of cell lysis that was observed with a 10-fold lower concentration of RBS when S° was not present (data not shown). To determine if the effect of RBS was mitigated by its direct reaction with S°, RBS (20 μM) was incubated with S° (1.0%, w/v S°) in the growth medium for 6 hours (the time needed for *P. furiosus* to reach mid-exponential growth) at 98ºC and then injected into a *P. furiosus* culture at a final concentration of 2 μM RBS. RBS remained as toxic as the untreated control, indicating that no reaction had occurred with S° (data not shown). To determine if *P. furiosus* cells had to interact with S° before S° had any effect on the toxicity of RBS, S° was added to cultures up to 30 minutes before the introduction of RBS (2μM). Only cells incubated with S° for at least 20 minutes at 98°C prior to the addition of RBS did not lyse and continued to grow (Figure 3.2B). To determine if insoluble particles other than S° provided any protection from RBS, *P. furiosus* cells were grown in the presence of excess iron sulfide (FeS, 0.1%, w/v). However, when added to the culture at a cell density of 5×10^7 cells/mL RBS still caused immediate cell lysis (data not shown). Consequently, unlike insoluble S°, the presence of insoluble FeS did not protect cells from RBS.

The optimal growth temperature of *P. furiosus* is near 100ºC and it shows reasonable growth at 72°C, with doubling times of approximately 1 and 5 hours,

respectively. There is no measurable growth at 4ºC where the metabolic activity approaches zero (4, 6, 7, 14, 28). To determine if rapid cell division was required for the toxic effects of RBS, cultures of *P. furiosus* were grown to a cell density of 5 x 10^7 cells/mL at 98ºC, cells were then transferred to 4ºC for 1-2 hours, and RBS (2.0 μM) was added (Figure 3.3). As observed at 98ºC, *P. furiosus* cells lysed immediately upon the addition of RBS. The experiment was repeated with cells grown in the presence of S° (0.1%, w/v). In this case, RBS (2.0 μM) had no effect on cell morphology and did not cause lysis, as was also observed with the culture grown at 98°C (data not shown).

The morphological response of *P. furiosus* to RBS was examined by scanning electron microscopy (SEM). A culture was grown to a cell density of 5 x 10^7 cells/mL, RBS (1.0 μM) was added, and cell samples were taken. As shown in Figure 3.4, the membranes of cells appeared to be rapidly disrupted by RBS, as indicated the presence of cellular debris within seconds of RBS addition. To further investigate the cellular effects of RBS, membranes were harvested anaerobically from a culture of *P. furiosus* (400 mL) immediately after RBS (20 μM RBS) had been added. The membranes from the RBS-treated cells, which will be termed RBS-treated membranes, were washed with buffer to remove any residual traces of RBS. Remarkably, the washed, RBS-treated membranes were toxic to *P. furiosus* cells as they caused lysis when added to a culture growing at 98°C. Membranes prepared from untreated cells (that had not been exposed to RBS) had no effect on cell growth. Assuming that the RBS-treated membranes absorbed all of the RBS that was added to the original culture (where RBS was added to a 400 ml culture to a final concentration of 20 μM), the maximum concentration of RBS in the membrane

prep was ~3.2 mM and 40 µl of this added to the second culture resulted in cell lysis. Since a concentration of 2 µM RBS causes cell lysis, one can calculate that the membranes of *P. furiosus* in the original culture absorbed approximately 16% of the RBS that was added. *P. furiosus* membranes therefore appear to have a very high affinity for RBS. This experiment was repeated using nitrite (800 µM) or NO (40 µM) in place of RBS. However, membranes isolated from cells treated with these compounds had no effect on growing *P. furiosus* cells (data not shown). On the other hand, when RBS-treated membranes obtained from *P. furiosus* cells treated with RBS (20 µM) were incubated with S° for 30 minutes, about fifteen times as much of the membrane material was required to cause lysis of a fresh culture. The calculated concentration of RBS in the membrane preparation from S°-exposed cells was equivalent to ~30 µM. It appeared, therefore, that incubation with S° for 30 minutes caused RBS to be lost (in the subsequent wash) from RBS-treated membranes, such that they contained only ~7% of the RBS that was absorbed in the absence of S°. When polysulfide (4 mM) was used in place of S°, it did not minimize the toxic effect of RBS-treated membranes (Figure 3.5).

The effect of RBS on the integrity of the membranes of *P. furiosus* was further investigated using a fluorescent LIVE/DEAD assay, where cell death is indicated by a dramatic loss of fluorescence in the presence of the reagent, Figure 3.6 (Molecular Probes, Eugene, OR). *P. furiosus* cells were grown in the presence and absence of S°. Surprisingly, both types of cell exhibited a dramatic loss of fluorescence when RBS was added. However, microscopic analyses confirmed that cells grown without S° did indeed lyse, yet those grown with S° did not.

Discussion

The mechanism of action of RBS has remained a mystery for almost 150 years. NO has always been suspected to play a role since each RBS molecule contains seven NO ligands (1, 2, 6, 11, 17, 18, 20-23, 30). What is known of the antimicrobial interactions of RBS was revealed by extensive work with organisms from the genus *Clostridium* (1, 11, 17, 18). Other gram positive and also gram negative organisms are sensitive to RBS to varying degrees (17). Aerobic photolysis of RBS in the presence of endothelial cells leads to the rapid release of NO causing vasodilation, but RBS does not appear to have any other effect upon eukaryotic cells (21, 22, 30).

There is no evidence that NO is involved in the toxic effect of RBS under anaerobic conditions (6). In fact, NO is not released even when RBS is incubated anaerobically at 98ºC, and the compound appears to be stable under such conditions. This implies that NO is not involved in the cytotoxic mechanism of RBS and that its effect is due to other properties. *P. furiosus* is lysed by an RBS concentration of only 2 μM, as compared to 0.5 μM to inhibit the growth of vegetative cells of *C. perfringens*, 3 μM for *Listeria monocytogenes*, and 1.3 μM *C. sporogenes* (1). Lysis of *P. furiosus* cells by RBS occurs both near the optimum growth temperature, 98°C, but also occurs at 4°C, where metabolic and enzymatic activities are effectively zero. The toxic effects of RBS are therefore unlikely to arise from a product of its metabolism. Our results also clearly indicate that RBS is targeted to the cell membrane. Moreover, it appears to be absorbed into the membranes in an unmodified form, since membranes from RBS-treated cells are toxic to cultures not previously exposed to RBS. In contrast, even though nitrite and NO

cause *P. furiosus* cells to lyse, the same treated membranes had no effect on fresh cultures. Scanning electron microscopy (SEM) confirmed that the membrane is indeed compromised by RBS. It is therefore concluded that RBS toxicity is not dependent upon NO release, rather, it is due to a physical interaction with the cell membrane. Indeed, we were unable to obtain meaningful compression isotherms from lipid monolayer studies when RBS was present in the aqueous phase (data not shown) (31). In fact, we have previously used RBS and assumed it to be a NO generator, showing that *P. woesei* is more sensitive to this compound than *P. furiosus* (25). In light of the results presented herein, it would appear that the membranes of *P. furiosus* are more resistant to disruption by RBS, than are those of *P. woesei,* consistent with what has been previously reported by others (32).

The toxicity of RBS was dramatically reduced when *P. furiosus* cells were grown in the presence of S°. In fact, S° appeared to cause membranes from RBS-treated cells to lose 93% of the RBS that they had absorbed, yet, there appeared to be no chemical reaction between RBS and S°. The effects of insoluble S° on RBS toxicitycould not be reproduced with soluble polysulfide. It can therefore be hypothesized that insoluble S° is able to 'dissolve' into the cell membrane at 98°C and, by an as-yet unknown mechanism, prevent the effects of RBS. This also suggests that S^o is not actively transported into the cell, which is consistent with the absence of any S^o-regulated transporter as determined by DNA microarray analyses (33-37). Presumably S° interacts with the cell membrane in such a way that it competes with RBS, while higher concentrations of RBS can in turn outcompete S°. Fluorescence and microscopic analyses confirmed that RBS causes cell

death by lysis. Even though addition of RBS to S°-grown cells does not cause cell lysis or even loss of viability, the fluorescence-based LIVE/DEAD assay showed that there was a loss of membrane integrity similar to that seen with lysed cells. Presumably, RBS partially compromised the cell membrane of S°-grown cells although the mechanism is not understood.

Antimicrobial agents that compromise membrane integrity by forming pores or by lowering surface tension between the lipid and water phases have been known for some time (33-35, 37). RBS appears to have a similar mechanism of action and might be thought of as the first example of an inorganic chemical surfactant that functions as an antimicrobial agent (33). In addition, we show here that the thermo stability of RBS is high enough for its use with hyperthermophilic archaea growing at the normal boiling point of water. We also show, almost 150 years after its discovery, that the anaerobic mechanism of action of this enigmatic molecule has nothing to do with NO, but is due instead to its destructive interaction with prokaryotic membranes.

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Figure 3.1: Effect of RBS on the growth of *P. furiosus.* The concentrations of RBS are as follows: 0 μM (▲), 0.5 μM (■), 1.0 μM (♦), 2.0 μM (Δ), 3.0 μM (○) and 5.0 μM (□). The arrow indicates when RBS was added.

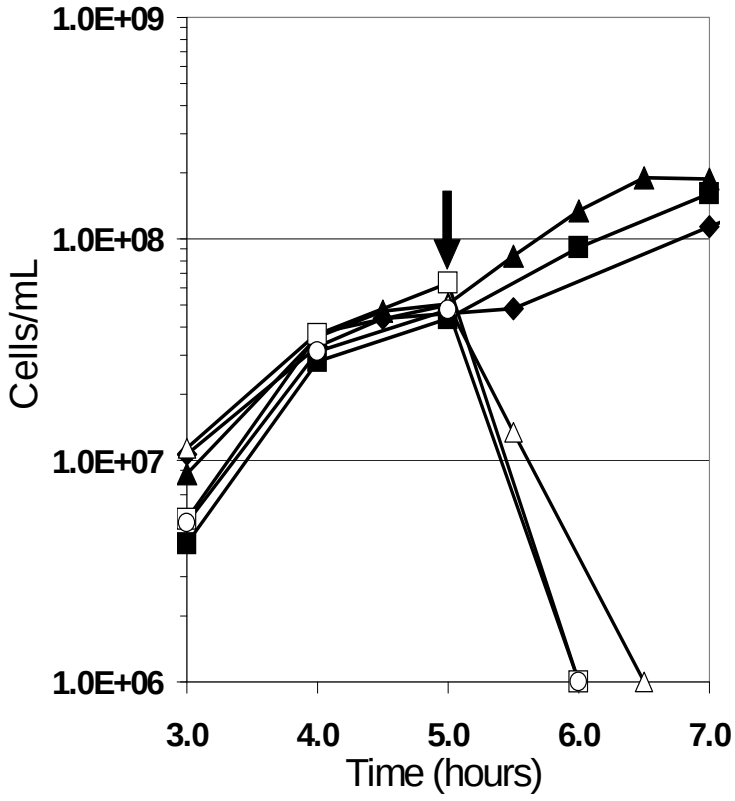


Figure 3.2A: Effect of RBS on *P. furiosus* grown in the presence of S°. The symbols represent: no S° (●), Sº added but no RBS (■), no Sº plus 2 µM RBS (Δ), Sº added plus 2 µM RBS (▲). The arrow indicates when RBS was added.

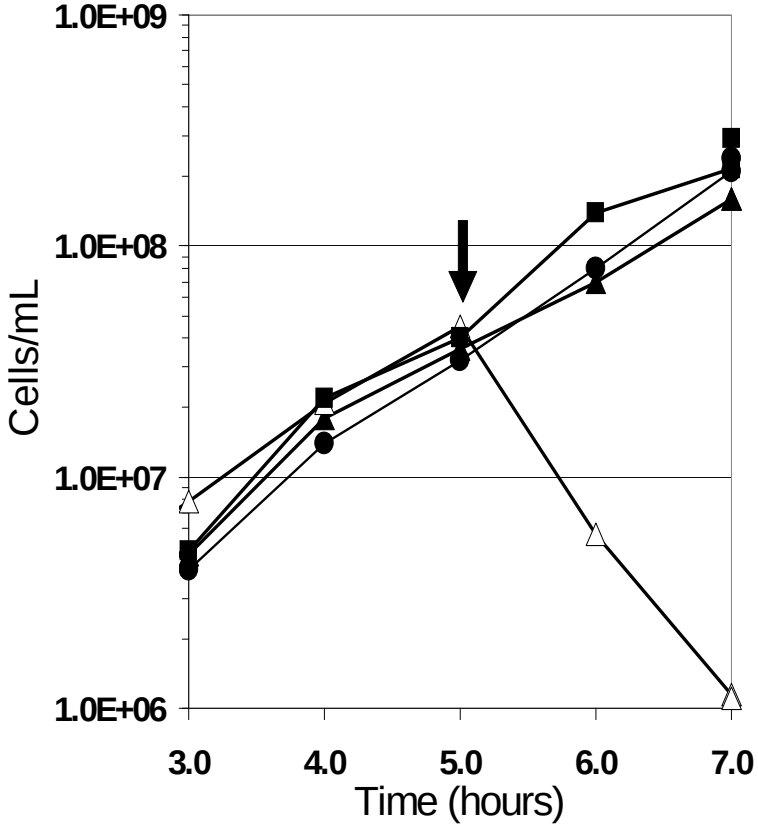


Figure 3.2B: Effect of RBS on *P. furiosus* after incubation with S°. The symbols represent: no Sº without RBS (▲), Sº and 2 µM RBS introduced to the culture at the same time (Δ), 2 µM RBS added after culture was incubated with S° for 10 minute (◊), 2 µM RBS added after culture was incubated with S° for 20 minute (○), 2 µM RBS added after culture was incubated with S° for 30 minute (■). The arrow indicates when RBS was added.

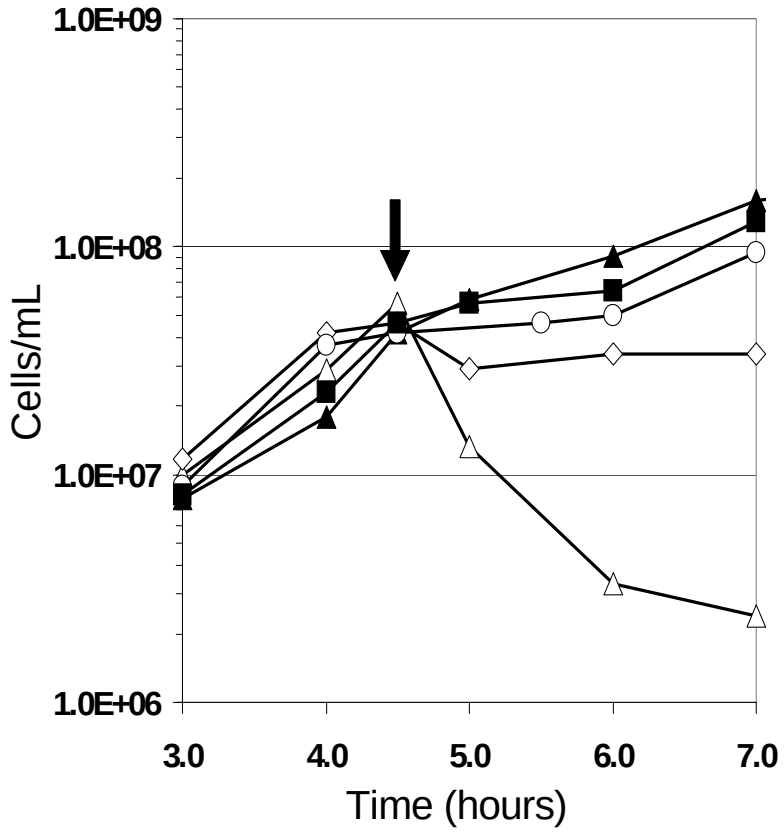


Figure 3.3: Toxicity of RBS at 4ºC. *P. furiosus* cultures were transferred from 98ºC to 4ºC after 4 hours (indicated by the first arrow) except the control which was maintained at the optimal growth temperature of 98°C. RBS was added to a culture once equilibrated to 4ºC at the 6 hour mark (indicated by the second arrow). The symbols represent: culture maintained at 98°C and no RBS added (▲), culture transferred to 4ºC but no RBS added (□), culture transferred to 4ºC and 2.0 µM RBS added (Δ).

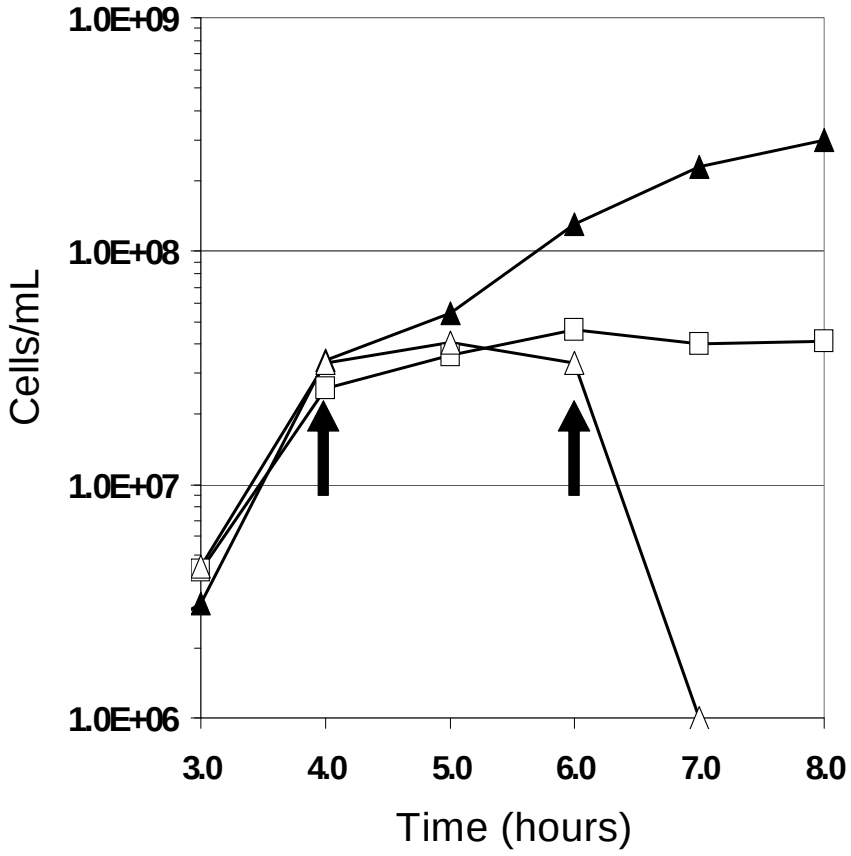


Figure 3.4: SEM images of *P. furiosus* exposed to 1 µM RBS. Images were collected before (A), and 60 seconds after (B) the addition of a non lethal dose of 1 µM RBS. The magnification is 10,000x.

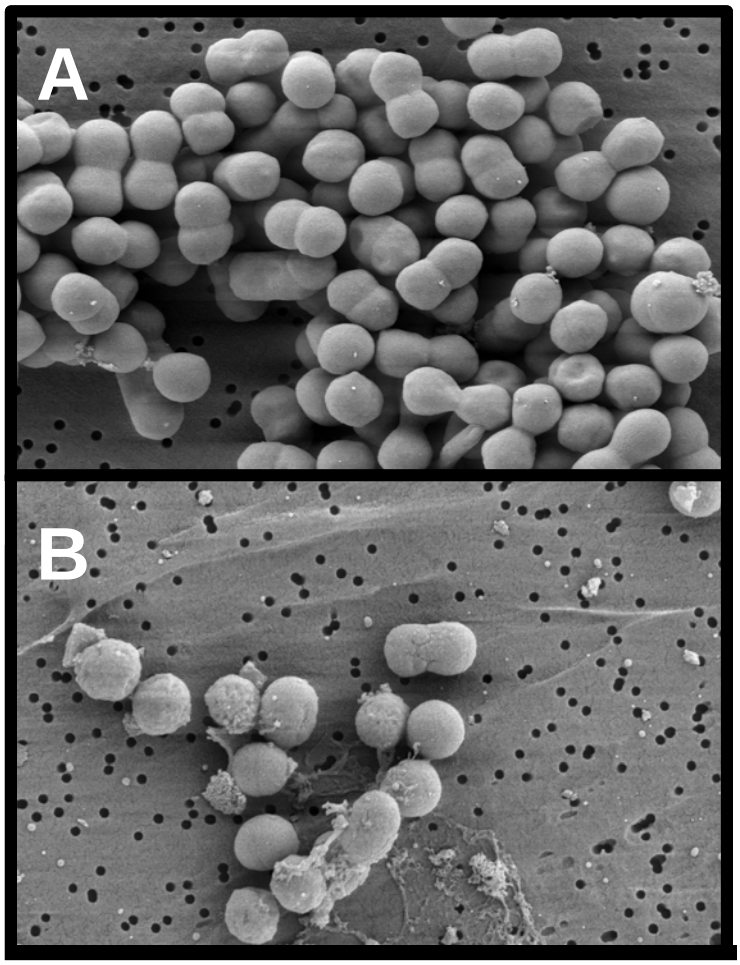


Figure 3.5: Transfer of RBS toxicity in *P. furiosus* membrane fractions. Membranes from cultures treated with 20 μM RBS were added to fresh *P. furiosus* cultures with increasing injection volumes (μL). Each culture was monitored by cell count after 30 minutes incubation with the RBS/membrane preparation. The symbols represent: no membranes added (■), RBS-treated membranes added (●), RBS-treated membranes added after prior incubation with S° (~4mM) at 98°C for 30 minutes (♦), RBS-treated membranes added after prior incubation with polysulfide (4 mM) at 98°C for 30 minutes (▲). Cultures with cell densities below 1 x 10^6 cells/mL were considered not viable.

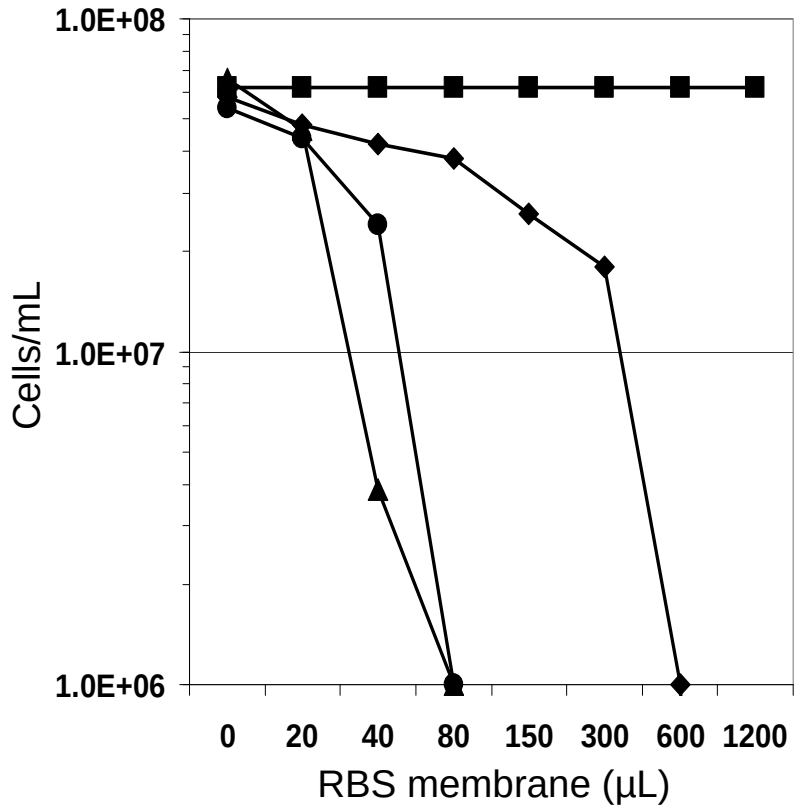
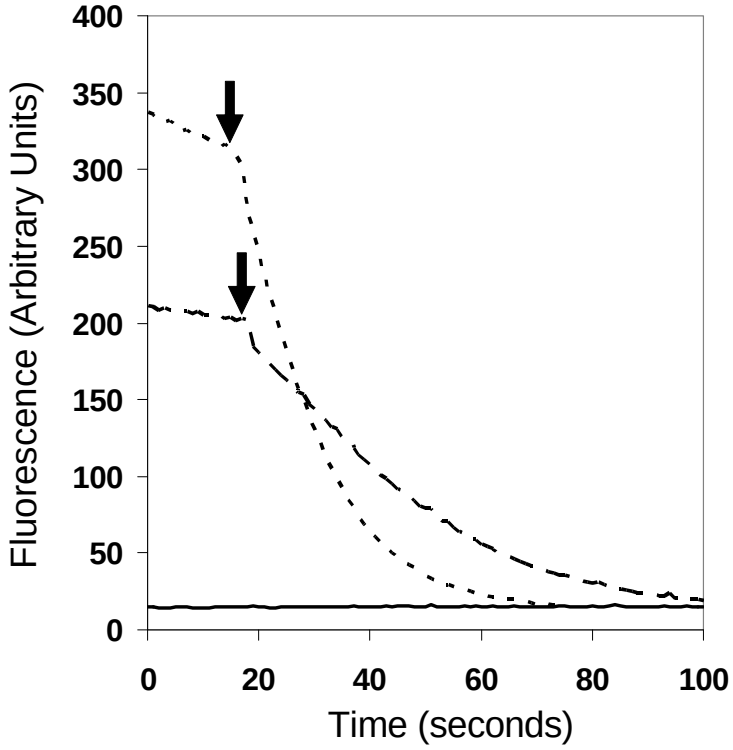


Figure 4.6: Fluorescent LIVE/DEAD Assays on *P. furiosus* cells treated with RBS. The cell concentrations were initially 5.0 x 10^7 cells/mL. RBS (2 μM) was injected approximately 20 seconds after beginning recording of fluorescent scan. The symbols represent; *P. furiosus* cells grown without S° (····), *P. furiosus* cells grown with S° (----), control without cells (—).



CHAPTER 4

SUMMARY AND DISCUSSION

Advances in DNA microarray technology have made this a powerful technique that can be adapted to a wide variety of research applications. Whether monitoring changes in RNA transcript levels in response to stress, comparing sequence similarity between genomes, or direct environmental analyses, the microarray technique has expanded to be a multitasking, high throughput approach. From humble beginnings as the Southern blot technique, microarray procedures have evolved and caused a revolution in silane surface chemistry, fluorophores, equipment for hybridization, fluorescent scanners, software packages and linking chemistry. The 2007 annual earnings reported by Affymetrix on microarray related products and services were $350 million. With a growth of 11% annually, it is expected that microarray earnings will exceed $1 billion per year by the year 2010 (www.marketresearch.com). Not only is microarray a revolutionary technique, it has driven a major industry creating many jobs for researchers, developers and science marketing.

The most profound advances in microarray technology have involved new chemistry that link fluorophors with target oligonucleotide probes. Initially the process relied upon a reverse transcriptase or a DNA polymerase to label RNA (and make a DNA copy) and DNA, respectively, which were inefficient at incorporating primary or secondary labels into the

produced probe. A new innovative product has been developed using a direct label upon the isolated RNA or DNA using a platinum tag that has a fluorophore of choice connected to it (Molecular Probes, Eugene OR). The platinum tag is covalently attached to the 7th nitrogen of guanine. This makes the labeling process faster, it allows direct labeling of RNA and DNA, and even environmental samples that were, prior to this product, too low in concentration to be used with the enzyme based labeling method (see Appendix A).

The versatility of the microarray technology allows for analysis of transcripts of an organism reacting to an environmental stress or as a genomic comparative analysis. *P. furiosus* has provided a benchmark for hyperthermophile research. *P. furiosus* microarrays have revealed much about the metabolic functionality of many ORFs (28-30, 33, 34). The relationship between *P. furiosus* and *P. woesei* was shrouded in uncertainty as the similarities or differences were contested yet never explored in detail (15, 36). The microarray technique proved to be the tool to enable genomic comparisons between *P. furiosus* and *P. woesei*. One, disadvantage to this technique is that the *P. furiosus* microarray is only able to detect what is on the array. If, for example, *P. woesei* possesses genes for which *P. furiosus* does not have a homolog, these would not be detected on the array. The microarray technique readily shows how many of the *P. furiosus* genes have homologs in *P. woesei,* and the results can be easily validated by sequencing.

Hydrothermal ecosystems on the shores of the island of Vulcano, Italy, harbor a great resource of thermophilic life. Thermophilic communities are believed to involve a dynamic genetic exchange between organisms, even across the domains bacteria and archaea, through the

process of LGT. This is clearly evident when the genome sequences of two organisms from Vulcano, one a bacterium, *Thermotoga maritima* and one an archaeon, *P. furiosus*, were compared (19, 21-24). Almost one-quarter of all *T. maritima* ORFs had best matches to archaeal species. Many of the ORFs were arranged into groups or 'islands' that had matches to archaeal species. Among hyperthermophilic archaea, LGT events appear to have occurred between *P. furiosus* and *Thermococcus litoralis* (9). It is possible that the Vulcano island vent community is in a continuous state of competency to receive and transfer genetic information as the marine waters contain fluctuating concentrations of Mg^{2+} and Ca^{2+} ions, species that are comparable to those that are used to make *Escherichia coli* competent (1, 35). If the environment promotes genetic promiscuity this may be an explanation of how the LUCA emerged and diversified when the availability of genes was limited and innovation was needed if organisms were to spread through different communities rapidly. What was revealed by the comparisons between *P. woesei* and *P. furiosus* were distinct groups of ORFs that were seemingly missing from *P. woesei*. These groups of gene were flanked by ISs or LCTRs. As mentioned in Chapter 2 ISs have been associated with LGT, in most cases the missing ORF clusters of P. woesei are located in the P. furiosus genome in close proximity to ISs. What was also interesting to see flanking some of the missing gene clusters are the LCTR's, later known as clustered regularly interspaced short palindromic repeats (CRISPR) (3). CRISPRs are believed to be involved with resistance to phage infections. The CRISPRs contain the markers from prior phage exposures, possibly events of LGT driven by viral infection. It then would not be unexpected to find CRISPR sequences aligned with missing ORF clusters.

Of the metabolic consequences due to the missing ORFs the inability to transport maltose and tolerance to nitric oxide were assayed. It was later discovered that NO tolerance was not the appropriate test as the putative NO generator RBS does not produce NO (see Chapter 3). Nevertheless, the differential response of *P. woesei* and *P. furiosus* to RBS can be explained by the fragile membrane structure of *P. woesei* (15). A re-examination of the 105 *P. furiosus* genes that are "missing" in *P. woesei* accounted for 25 ORFs with ≥2 predicted trans-membrane domains. The loss of membrane stability without these proteins might be the direct cause of why *P. furiosus* compared to *P. woesei* in the presence of RBS was the result of these ORF not present in *P. woesei*.

The antimicrobial properties of RBS have remained a mystery for 150 years. In 1858 iron-sulfur nitrosyl salts were described by the French scientist M. L. Roussin. In 1967 it was discovered that when nitrite is heated (> 60˚C) with ammonium polysulphide and $FeSO_4$, a dark precipitate is formed which is many times more potent than nitrite to species of *Clostridium* (25, 26). These products were referred to as "Perigo type factors" (14, 17, 25, 26). In 1974, it was found that an iron-sulfur nitrosyl salt is the active molecule in the "Perigo type factors" (2). Named after the initial discover, Roussin, the molecular structure of RBS is $Fe_4S_3(NO)_7$ with seven moles of NO positioned around the iron-sulfur core. Under aerobic conditions the molecule breaks down over several days decomposing into iron, sulfur and nitric oxide (7, 17, 18, 25). While in an aerobic environment and exposed to high energy light RBS will decompose releasing NO over a time interval of seconds (5, 10). Decomposition of RBS while maintained under an anaerobic environment does not release NO (5, 12, 17).

NO has been suspected to play a role in the mechanism of RBS toxicity (8, 13, 14, 17, 20, 25). However, when incubated anaerobically up to a temperature of 60ºC, RBS showed no sign of producing NO (17, 27). In chapter 3 the temperature of 100°C, which is the physiological temperature of *P. furiosus*, is used to determine if any NO is released through spectral and S-nitrosothiol formation. If NO is not being released under the conditions which the anaerobic *P. furiosus* grows, yet RBS retains its cytotoxic effects, the mechanism does not appear to require the production of NO. The caveat is that *P. furiosus* might metabolize RBS to give rise to an active RNS species. However, we found that when *P. furiosus* cells were transferred from 100°C to 4°C, they were still sensitive to the lytic effects of RBS. At 4°C it is unlikely that a hyperthermophile such as *P. furiosus* is able to metabolize RBS within the seconds of the observed lysis by RBS. The inability of *P. furiosus* to metabolize RBS was further supported by the fact that cell membranes collected from *P. furiosus* cultures lysed with 20 μM RBS were themselves toxic to fresh *P. furiosus* cultures that had not seen RBS. Only the membrane fragments retained the RBS cytotoxic effects, and not the soluble supernatant fraction that would be expected to contain a RBS-derived metabolite if it were produced. Cytotoxic transfers of cell pellets cannot be replicated with nitrite or NO, which further distinguishes the mechanism of RBS from that of other reactive nitrogen species.

The unique properties of RBS were further revealed when it was discovered that the lytic effects can be diminished when *P. furiosus* is grown on elemental sulfur (S°). *P. furiosus* can utilize S° as a terminal electron acceptor. DNA microarray and biochemical analyses have shown that S° induces the synthesis of a membrane complex termed MBX. MBX is encoded by ≥13 genes, which have homology to the membrane-bound hydrogenase (MBH) of *P. furiosus*

(29). MBX is proposed to oxidize ferredoxin and reduce NADP and conserve energy as a proton motive force. The NADPH is oxidized by a cytoplasmic flavoprotein (~100 kDa) termed NADPH sulfur reductase (NSR), which reduces S° to H_2S in a coenzyme A-dependent reaction (29). As shown in Figure 4.1, NSR is proposed as the primary S°-reducing enzyme in *P. furiosus*, although the mechanism by which S° enters the cell is unknown. The lytic effects of RBS are not reduced if the compound is mixed with a suspension of S° before injection into a culture. It was found that S° needed to be present in a growing culture at 98°C for at least 10 minutes before cells become resistant to the effects of RBS. Washed membranes isolated from cells lysed with RBS carry over the lytic effects to new cultures. If the washed RBS membranes are incubated with colloidal sulfur the lytic potency to a new cultures is lessened by 93% as compared to RBS membranes not treated with colloidal sulfur (Chapter 3). When the same experiment was repeated using polysulfide, there were no changes in the effects of RBS. Reduction in the effects of RBS is caused specifically by the presence of S° and does not rely on living cells to transport or metabolize the sulfur.

There is, therefore, a correlation between the presence of sulfur, membrane structure and the effects of RBS. It is not known if the resistance to RBS results from the physical presence of S° or of a metabolite of S° that causes a morphological change in the S-layer or in the membrane. As yet, the process by which S° is transported from outside the cell into the cytoplasm is not clearly understood. It is possible that there is an uncharacterized hydrophobic transporter involved or S° could passively diffuse into the membrane causing a membrane/S-layer conformation change (16). *P. furiosus* growth temperature is 100°C, which is very close to the melting temperature of S°, which is 105°C. Sulfur polymerizes forming typically 8 member

rings once in a liquid state (4, 11). It is possible that at the growth temperature, S° diffuses through and across the membrane, or it forms a molten-like state that readily diffuses or is accessible to a transporter. As mentioned before, S° does not react with RBS, but through ring formation or indirect up-regulation of *P. furiosus* membrane/S-layer ORFs the architectural structure and integrity of the membrane might be maintained so that the cell does not lyse due to RBS (see Figure 4.2). The membrane studies from Chapter 3 suggest that colloidal sulfur (not in a melted state) is sufficient to reduce the effects of RBS without inducing an S-layer effect. In any case, with increasing amounts of RBS, even the membranes of S° grown cells will lyse when shocked with high (20 μM) concentration of RBS. The effectiveness of RBS may be directly correlated to the stability and availability of prokaryote membrane structure. RBS is an effective bactericide against several species of *Clostridium,* both in the vegetative and the spore state (2, 6, 14, 20). Interestingly RBS has varying degrees of toxicity to Gram-negative and Gram-positive bacteria. From inhibitory growth experiments it was found that Gram-negative organisms are seemingly more sensitive (2). This observation might be explained by the presence of the substantially thicker peptidoglycan cell wall of Gram-positive bacteria which could provide a barrier or at least slow the progress of RBS. If we consider the results from Chapter 3, it appears that RBS preferentially adheres to lipid material and disrupts membranes when a critical concentration is reached. Organisms with thick peptidoglycan, structurally-enhancing transmembrane proteins, or thick S-layers would naturally be more resistant to RBS.

Molecules that can disrupt lipid structures are known as surfactants. These molecules accumulate at the interface between immiscible phases. By lowering the surface tension at interfaces hydrophilic and hydrophobic phases become miscible (31, 32). Like RBS, surfactants

have antimicrobial effects both in the vegetative and spore states. Most surfactants are produced by biotic means or are organically-synthesized to mimic biologically-derived molecules. Inorganic surfactants that have antimicrobial activity have not been reported. Moreover, no inorganic surfactant composed of an iron sulfur cluster center has been previously characterized. Consequently, RBS appears to be the first example of an antimicrobial inorganic surfactant.

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Figure 4.1: Sulfur reduction pathway in *P. furiosus*. This is the proposed pathway of electron flow during carbohydrate metabolism and S° reduction. Via ferredoxin, electrons are shuttled to the MBX complex and in a conserved step NADP is reduced, which then delivers the electrons to the cytoplasmic NADPH sulfur reductase. Adapted from (29).

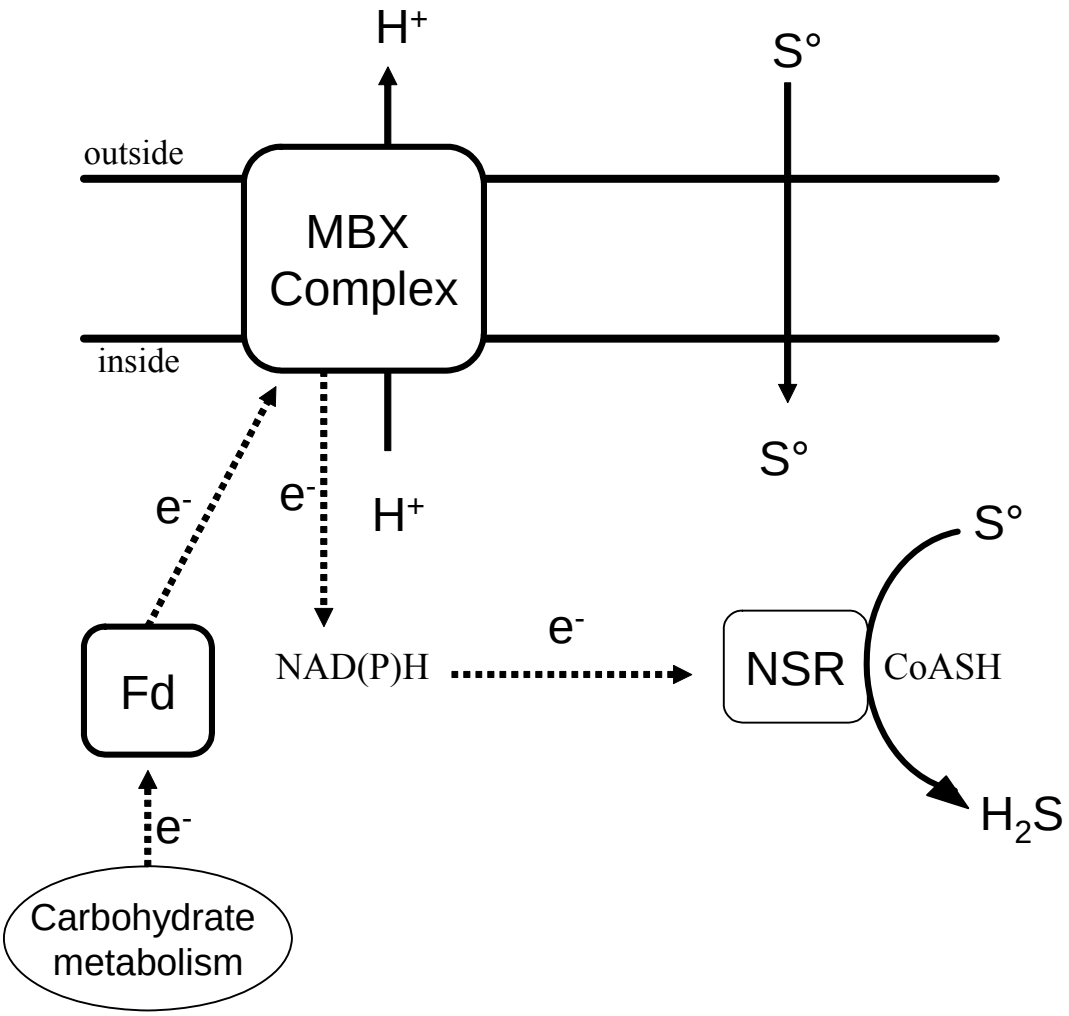
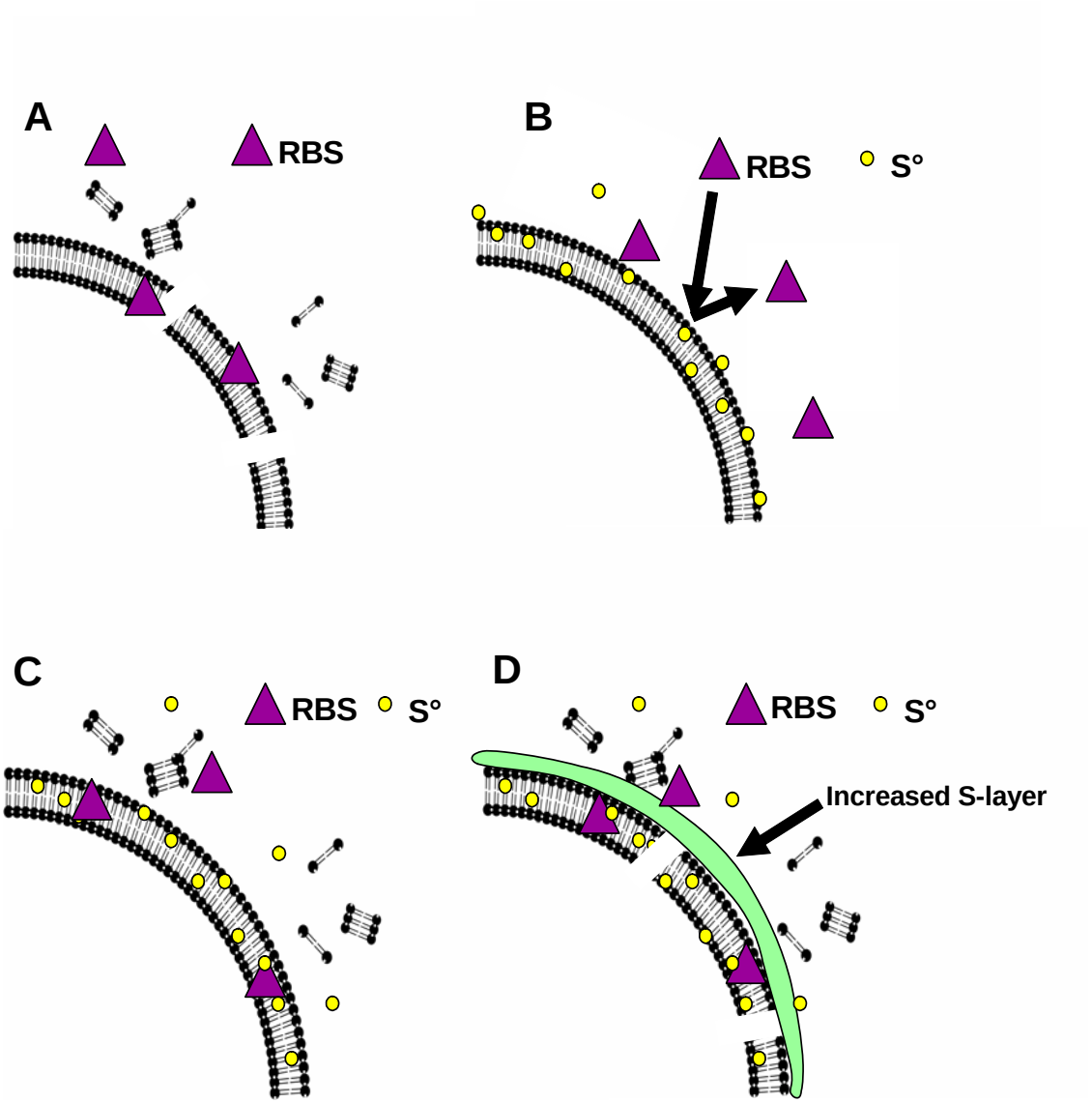


Figure 4.2: Proposed mechanisms for the antimicrobial action of RBS and of the resistance induced by S°. A) Membrane disruption and cell lysis by RBS, B) Cells grown on S° resist RBS C) S° allows cells to persists even in the presence of RBS, D) S° may cause an increase in membrane organization or S-layer production to facilitate S° passage into the cell for conservation of energy by reduction to H_2S which stabilizes cell membrane from RBS.



APPENDICES

APPENDIX A. DEVELOPMENT OF THE DNA MICROARRAY APPROACH FOR STUDIES OF *PYROCOCCUS FURIOSUS*

ABSTRACT

The DNA microarray technique has become a powerful molecular biology tool. It allows the quick and efficient monitoring of the RNA content of a microorganism or of a specific tissue and can be used to determine how the system responds to environmental changes. This technique can also be used for genomic comparisons between similar organisms. This chapter describes the development of this technique for studies of the hyperthermophile *Pyrococcus furiosus*. The current procedures are described in detail and include the methods for printing and blocking microarray slides, isolation of microarray-quality RNA and genomic DNA, direct and indirect fluorescent labeling, and hybridization.

INTRODUCTION

Since the introduction of the DNA microarray technique in 1995 it has proven to be a powerful tool in molecular biology and biochemistry (7). Its 'simple' design combines traditional molecular biology hybridization technology and the micron accuracy of robotic printing machines. The onset of the massive efforts to sequence the genome of organisms has provided the foundation with which to apply microarrays to monitoring whole genomes and metagenomes (1, 3, 8, 9). Together these advances have provided the ability to monitor thousands of transcripts from different tissues or single celled organisms on a massive high throughput level that was not possible before.

The microarray technique has undergone many revisions and improvements since its introduction. The microarray procedure requires two initial steps; one is the printing (by a robotic printer) of known genetic elements onto a solid support such as glass. The second part requires the isolation of RNA from the organism or the tissue. The RNA is then reverse transcribed into cDNA and labeled with a fluorescent probe. The fluorescently-labeled cDNA is then hybridized to the microarray grid containing printed DNA elements on the surface representing unique sequences. The fluorescent probes will hybridize to complimentary genetic elements on the microarray. When a fluorescent probe has hybridized to an element on the microarray grid, indicating a complimentary sequence, that location will fluoresce. The fluorescence can be measured and correlated with the genetic element that was printed at the location where hybridization occurred. The presence or absence of fluorescence at printed genetic elements indicates whether expression occurred for the particular gene of interest in the tissues or organism being studied. Thus whole genomes representing all expressed genes from

organism can be printed as a microarray and monitored. Alternatively, a genomic microarray can be used to analyze closely related organism as a measure of genomic relations.

In this document all aspects related to microarray design and fabrication, probe-labeling, hybridization, data analysis and troubleshooting are discussed in detail. In addition, recipe protocols, vendor contact information, product codes and troubleshooting sections have been included in an appendix. The microarray protocols described here were designed for the complete genome analysis of the hyperthermophilic archaeon *Pyrococcus furiosus*.

MICROARRAY DESIGN AND DEVELOPMENT

Generating the components for a microarray begins with producing PCR products from all 2192 open reading frames (ORF) in the *P. furiosus* genome (5)**.** Primers are designed to produce full length PCR products of ORFs from *P. furiosus*. They are designed using Array Designer software (Premier Biosoft International, Palo Alto, CA) for each ORF. In cases where the ORF was >2000 bp multiple primers was designed for screening purposes to produce a reliable PCR product. The primers are synthesized by Integrated DNA Technologies (Skokie, IL). The ORF products are generated in 96 well plates by mixing the PCR primer pairs, the Taq Plus Precision Polymerase as per manufacturer's directions (Stratagene, Herculese, CA). Genomic DNA from *P. furiosus* was isolated using a phenol/chloroform protocol (see Appendix D). PCR mixtures are thermo cycled for 30 runs using the temperature 56°C, 72°C and 95°C. PCR products are purified as per manufacturer's instructions using QIAquick 96 PCR Purification Kit (Qiagen, Valencia, CA) omitting the last step and replacing the kit elution solution with nuclease free water. Quality control of PCR products is performed by electrophoresis on 0.8% Agarose gels (Sigma, St. Louis, MO). Once the PCR products are purified and confirmed to have the expected size, the DNA elements are dried and dissolved in a mixture of 50% nuclease free water and 50% dimethyl sulfoxide (DMSO). The DNA elements are then loaded into 386 well plates (6 plates total in order to hold all *P. furiosus* 2176 ORFs). The position of each ORF is recorded in a tracking file by row, column and by 386 plate.

Using an Omni Grid printing robot (Genomic Solutions, Ann Arbor, MI) the PCR elements are printed onto a set of 70 solid support slides. The solid support on which the

elements are to be printed must be amino silane coated. Two suppliers of amino silane coated slides are MicroMax™ Super Chip™ I glass slides (Perken-Elmer, Waltham, MA) and Genorama® glass slides (Asper Biotech, Tartu, Estonia). Amino silane slides from both venders can be used in this printing procedure. The quills used in the robot are Stealth Micro Spotting Pins from ArrayIt™ Brand Products (Telechem International Inc., Sunnyvale, CA.) and operate by capillary action. Since the robot prints in sequential order, five 'dummy slides' or 'blotter slides' are placed at the beginning of the set to remove excess droplets from the printing pins as they are dipped into the loaded 386 well plates filled with the PCR products. The robot quill holder can hold up to 32 pins, but in this application we use only 8 pins arranged in a 2 x 4 grid (Figure A.1). The robot dips the pins into the 386 well plate and absorbs nanoliter amounts of the DNA PCR product. It then taps the pins onto the set of aminosilane glass slides arranged by rows on the printing stage. The quills are cleaned by nuclease free water and vacuum three times between each printing a set of 8 elements onto slides up to 70 slides. The location of the printed element onto the glass slide is tracked using a master list produced by the Genemachine software loaded into the robot's computer (Genomic Solutions). The tracking file lists which DNA element was printed at a given location on the microarray. This printing list must be aligned with the ORFs that were loaded into the 386 well plates.

The dimensions of the typical amino silane slide are 7.5 cm x 2.5 cm. The sub-array is 4.5 mm x 4.5 mm, which allows 4 sub-arrays to be printed along the width of a glass slide and 6 along the length covering an area of 4.9×10^8 microns^2. The arrangement of the *P. furiosus* array was designed to be printed as 2 rows x 4 columns (see Figure A.2). Within the print grid each sub-array consists of 19 x 19 elements. A total of 2192 elements can be printed once within

one basic print grid that can contain a maximum of 2888 elements (sub-arrays are 19 x 19 = 361 x 8 sub-arrays within basic print grid = 2888 elements total). The basic print grid is repeated 3 times on one silane slide. Thus three copies of the *P. furiosus* genome are represented on one slide.

Once the slides are printed, post-printing processing begins with hydrating the microarray surface. Typically the slides are stored in a slide box avoiding exposure to light and ambient air. Until post processing is complete the slides should only be removed from storage in a clean room (filtered positive air flow). A sterile bath of nuclease free water (200-300 mL) is heated to 50–65°C until water vapor can be seen rising from the water surface and the slides (array side facing towards the water vapor) are held over the rising water vapor for no more than 15 seconds, then removed. The slides are then allowed to dry on a heating block set at 80°C for approximately 5 seconds (array side up). The reason that the hydration is necessary at this point is to allow the spotted elements to spread into a uniform circle on the slide surface before the DNA is fixed to the solid support.

The association between the DNA elements and the slide is purely via charge-charge interactions (see Figure A.3). After hydration the slides must be covalently attached to the amino silane surface. There are two methods that can be employed to do this. One method uses heat while the other method uses UV light to crosslink the nucleotides to the slide surface. To induce cross linking by heat, the slides are arranged on a flat surface and covered with an autoclavable plastic lid, then placed in an oven at 85°C and incubated for 2 hours. After incubation, remove slides from oven and allow to equilibrate to room temperature for 10

minutes. After cooling wash slides profusely with nuclease free water to remove any unbound DNA. Dry slides in a microarray slide centrifuge rotor and store in a desiccated cool place. For cross linking by UV place a set of slides into a UV Crosslinker (Stratagene, Hercules, CA) and radiate the slides with 90 mJ. After UV cross linking wash slides profusely with nuclease free water to remove any unbound printed DNA. Dry slides in a microarray slide centrifuge rotor and store in a desiccated cool place. Alternatively, after covalently binding DNA to the slide, the slide can be washed instead with nuclease water that contains 1% w/v of SDS or v/v Tween-20 to assist with cleaning microarray slides with stubborn background anomalies.

The last step in post processing involves blocking the exposed areas of the amino silane slides that do not have DNA elements printed on the surface. Blocking can be accomplished by two methods: chemical modification or incubation with salmon sperm DNA (see Appendix B). Chemically-modifying the slide surface requires dissolving 3.2 g of succinic anhydride (Sigma, St. Louis, MO) into 200 mL of 1-methyl-2-pyrolidionone (Sigma, St. Louis, MO) and adding 22.2 mL of 0.2 M sodium borate (Sigma, St. Louis, MO) at pH 8.0 (see Figure A.4). The slides are then dipped 5 times and then incubated in the succinic anhydride solution for 5 minutes at 25ºC. After incubation, the slides are transferred to 95°C de-ionized distilled water for 2 minutes. Slides are then washed with 95% ethanol and dried by a Spectrafuge-Mini with a slide rotor (Labnet International Inc., Woodbridge, NJ). An alternative blocking procedure is to incubae the slides in a pre-hybridization solution of 2X sodium chloride/sodium citrate buffer (300 mM sodium chloride (final conc.), 300 mM sodium citrate) (SSC), 0.1% sodium dodecyl sulfate (SDS) and 0.1 mg/mL salmon sperm DNA (Sigma, St. Louis, MO) for 20 minutes at 25ºC. The slides are then washed 2-3 times with sterile nuclease free water (Sigma, St. Louis,

MO). After either blocking procedure, the slides are stored in a secure, desiccated location that is not exposed to direct sunlight.

Isolation of pure total RNA from *P. furiosus* can be accomplished by two methods, extraction by phenol: chloroform (see Appendix C for protocol) and using the Promega Midiprep System (http://www.promega.de/tbs/tm253/tm253.pdf, Promega, Madison, WI). Whether using either method, all plastic surfaces need to be treated with RNAse ZAP (Ambion, Austin, TX). The phenol: chloroform procedure for isolating total RNA from *P. furiosus* is adapted from a published protocol (6). A *P. furiosus* culture (3 liters, minimum cell density of 5.0 x 10^7 cells/mL for *P. furiosus*) is centrifuged at 4500 x *g* for 20 minutes at 4˚C. The supernatant is decanted and the pellet is re-suspend in 1.0 mL of ice cold 3% w/v NaCl. From this point on all extractions can be done at 25ºC. To the suspension add 3.0 mL of cell lysis buffer (4.0 M guanidine thiocyanate, 0.83% N-lauryl sarcosine, 30 mM Na-acetate pH 5.0, 700 µM β-mercaptoethanol, also see Appendix I) and 3.0 mL phenol: chloroform 5:1 acid equilibrated at pH 4.7 (Sigma, St. Louis, MO) mix solution and centrifuge for 20 min at 2000 x *g*. Remove and keep the upper aqueous phase then extract again with 3.0 mL phenol/chloroform 5:1, acid equilibrated pH 4.7 then centrifuge for 20 min at 2000 x *g*. Extract the aqueous layer one more time with phenol/chloroform then centrifuge for 20 min at 2000 x *g*. Aliquot the aqueous layer into 0.9 mL volumes, in each tube precipitate the RNA with 1.0 mL isopropanol (Sigma, St. Louis, MO). RNA samples are then stored at -80ºC indefinitely until needed for labeling. Samples are further purified for microarray labeling by centrifuging crude RNA samples for 20 min at 16,000 x *g*. The supernatant is discarded and the pellet is dissolved in 100 µL RNAse free water (Sigma, St. Louis, MO). Once the crude RNA is dissolved in water 500 µL of cell lyses

buffer and 600 µL phenol/chloroform 5:1, acid equilibrated pH 4.7 also added and mixed thoroughly. Once mixed, the RNA samples are kept at 25ºC and are centrifuged for 20 min at 16,000 x *g*. The water phase is removed and extracted with 600 µL chloroform (Sigma, St. Louis, MO). Samples are then centrifuged for 20 min at 16,000 x *g*, the water layer is removed, 600 µL of isopropanol is added and they are stored at -20˚C for 1 hour. After freezing, the samples are centrifuged for 20 min at 16,000 x *g*, the supernatant is decanted and the pellet is washed with 1.0 mL cold 70% ethanol. The samples are then centrifuged for 20 min at 16,000 x *g*, the supernatant is completely removed and then the RNA pellet is dissolved in 100 µL absolute RNAse free water. The end concentration is determined by spectrophotmetry (see below).

RNA isolation by the Promega PureYield™ RNA Midiprep System has been adapted from the Promega kit protocol (http://www.promega.de/tbs/tm253/tm253.pdf). The volume of the *P. furiosus* culture used was 1000 mL (minimum cell density of 5 x 10^7 cells/mL). Cells are pelleted by centrifugation at 4500 x *g* for 20 mins. The supernatant is discarded and resuspend the cell pellet in 1.0 mL 3% w/v NaCl. To the suspended cells the following is added: 1.0 mL Lysis solution (from the Promega kit), 4 mL of RNA Dilution Buffer (Promega kit), 1.0 mL Clearing Agent (Promega kit), and the samples are mixed thoroughly. The mixture is incubated at 70ºC for 5 minutes and then cooled to 25ºC for 5 minutes. The lysate is mixed vigorously and poured into a BLUE PureYield™ clearing column (Promega kit). Centrifuge the clearing column for 10 minutes at 2000 x *g*, add 4 mL of isopropanol to the effluent and pour into a PureYield™ binding column (Promega kit). Centrifuge the column for 10 minutes at 2000 x *g* and then discard the flow-through. Add 20 mL of RNA Wash Solution (Promega kit) to the

column and centrifuge again for 5 minutes at 2000 x *g*. Discard the flow-through and 10 mL of RNA Wash Solution to the binding column. Centrifuge the sample for 10 minutes at 2100 x *g*, discard flow-through and elute the RNA from binding column with 1.0 mL nuclease-free water.

Isolation of genomic DNA from *P. furiosus* is needed to generate template for the PCR reaction, and it is also used as a positive control for the microarray. Genomic DNA can be isolated from *P. furiosus* using an adapted phenol: chloroform method (see Appendix D for protocol) (2, 4, 6). A *P. furiosus* culture (800 mL at a cell density of >1.0 x 10^8 cells/mL) is centrifuged at 4500 x *g* for 15 minutes at 4°C. Resuspend the pellet in 5 mL of chromosomal DNA isolation cell resuspension buffer (25% w/v sucrose, 50 mM Tris at pH 7.8, 40 mM EDTA, also see Appendix J). To the cell suspension add 50 µL of 20 mg/mL Proteinase K (Sigma, St. Louis, MO) in nuclease free water, 125 µL of 10 mg/mL RNAse (made DNAse free by boiling sample for 20 minutes) and 2.0 mL 0.5 M EDTA at pH 8.0. Incubate suspension at 37°C for 1 hour and increases the volume to 6 mL with 10 mM Tris-EDTA (TE) buffer at pH 8. While gently mixing the following is added to the sample: 1.25 mL of 10% w/v SDS, 0.45 mL of 25% v/v Triton X-100, 1.35 mL of 5.0 M NaCl and 1.25 mL of 10% w/v hexadecyltrimethyl ammonium bromide (CTAB) (Sigma, St. Louis, MO). Mix thoroughly by inversion (do not vortex) and incubate at 65°C for 45 minutes. Add an equal volume (approximately 10 mL) of 25:24:1 phenol: chloroform: isoamyl alcohol, mix, then centrifuge the samples for 20 minutes at 3000 x *g* at 4°C. The aqueous layer is removed and extracted 3 more times with 25:24:1 phenol: chloroform: isoamyl alcohol. The final centrifugation is carried out at 3000 x *g* for 20 minutes at 4°C. Extract the sample with an equal volume of chloroform and centrifuge at 3000 x *g* at 4ºC. Remove the aqueous layer and add 1/10 volume of 5 M NaCl. DNA is precipitated by adding

two volumes of 100% isopropanol and mixing gently. Genomic DNA is removed from the isopropanol with a sterile glass 'J' Pasteur pipette. Transfer the DNA directly to 20 mL of 70% v/v ethanol and gently wash by rocking. Centrifuge the DNA at 3000 x *g* for 15 minutes. Decant the ethanol and allow the pellet to dry for 5 minutes. Dissolve the pellet in 100 µL of 10 mM TE buffer at pH 8. Determine the concentration of DNA by 260 nm absorbance (as ascribed below).

Determining the concentration of RNA and DNA is accomplished by spectrophotometry (6). The process requires only a 5.0 µL sample of RNA or DNA solution which then is diluted with nuclease free water to a volume of 500 µL in a quartz cuvet. The absorbance is measured at 260 nm and 280 nm. To determine concentrations, for RNA a A_{260} value of 1.0 corresponds to 40 µg/mL, and for dsDNA, a A_{260} of 1.0 corresponds to 50 µg/mL (6). The purity can be determined through the ratio of A_{260}/A_{280} for DNA and RNA. Ratios of 1.8 and 2.0 for DNA and RNA, respectively, are optimal for microarray labeling (6).

Once pure samples of RNA and DNA are obtained, in order to be used as probes on the microarray they have to be labeled with a fluorescent molecule. Two methods of fluorescent labeling have been optimized, direct (see Appendix E for protocol) and indirect labeling, adapted from the manufacturer's procedures (see Appendix F for RNA and G for DNA protocols) (Molecular Probes, Eugen, OR). The direct labeling procedure uses the ULYSIS™ Alexa Fluor® labeling kit while the indirect method uses the ARES™ Alexa Fluor® labeling kit. The direct labeling method using the ULYSIS™ Alexa Fluor® kit (see Figure A.5) and requires only 5 µg RNA or 1 µg DNA (in volumes no greater than 5 µL). Mix the nucleotide sample with 20 µL

Labeling buffer (component C from ULYSIS kit) and incubate for 5 minutes at 65ºC (for RNA) or 95ºC (for DNA). After incubation place the sample on ice immediately and allow to equilibrate. Mix the sample (volume 25 μL) with 4.0 μL ULYSIS fluorescent label (Alexa dye 546nm, 596nm and 647nm) or 1 μL ULYSIS fluorescent label for ULYSIS Alexa 488nm (as per manufacture's directions). Incubate RNA sample(s) for 10 minutes at 90ºC and DNA sample(s) for 15 minutes at 80ºC, and after incubation plunge the samples into an ice bath. Prepare Micro Bio-Spin® 30 purification columns (Bio Rad, Hercules, CA) as per manufacturer's directions for a buffer-less preparation and apply labeled sample to the center of the column. Centrifuge column for 4 minutes at 1000 x *g*. Keep the flow-through and then vacuum dry in a centrifugal evaporator at 1000 x *g* and 25ºC (Jouan, Winchester, VA) until approximately 1 μL is left (approximately 30 minutes). The direct labeling method uses the ARES™ Alexa Fluor® labeling kit (see Figure A.6). In a centrifuge tube add 40 μL of 27 OD/mL random 9-mer primer (Stratagene, La Jolla, CA) then vacuum dry random primer in a centrifugal evaporator (Jouan, Winchester, VA). To the dried primer add 25 μL of target RNA (concentration of 2 μg/μL) and fill to a total volume of 35 μL with nuclease free water. Incubate sample at 60°C for 15 minutes then immediately inject 1.0 μL RNAse Block and allow the sample to equilibrate to 25ºC. To the sample add 2.5 μL of 25 mM $MgCl_2$ (Stratagene, La Jolla, CA), 10 μL of 10X Reverse Transcriptase Buffer (Stratagene, La Jolla, CA), 10 μL of 10 mM dCTP, dGTP (Sigma, St. Louis, MO), dATP/0.5 mM TTP (Sigma, St. Louis, MO), 20.0 μL of 2 mM aminoallyl dUTP (Sigma, St. Loui, MO) and 5.0 μL of Stratascript RT (50 U/mL) (Stratagene, La Jolla, CA.). Incubate for 30 minutes at 37°C then add 3.0 μL of Stratascript RT (50 U/mL) and continue incubation for 30 minutes at 37°C. Add 11 μL of 1.0 M NaOH then incubate the sample for 8 minutes at 60°C. Adjust the pH using 20 μL of 2.4 M 3-Morpholinopropanesulfonic acid

(MOPS) pH 4.0 so that the resulting pH is lower than 7.0. Heat denature the sample at 90°C for 2 minutes and mix with 500 μL Qiagen Binding Buffer (Qiagen, Valencia, CA). Add the sample to purification spin columns and centrifuge for 1 minute at 1,300 x *g*. Wash the sample bound on centrifuge column with 100 μL of Qiagen Binding Buffer and centrifuge for 1 minute at 1,300 x *g*. Wash the sample twice with 500 μL of 80% ethanol buffered with Qiagen's proprietary solution (Qiagen, Valencia, CA). The last spin should be faster (16,000 x *g*) to make sure all ethanol is removed. Elute labeled cDNA 3 times with 20 μL of nuclease free water into sterile 1.5 mL microfuge tubes (allow the first 20 μL to soak into spin-column for 5 mins before centrifugation). The labeled cDNA is in the water elution. Speed vacuum sample in centrifuge evaporator at 1,000 x *g* and 25ºC (Jouan, Winchester, VA) until approximately 1 μL is left (approximately 30 minutes). Add 5.0 μL of nuclease free water and 3.0 μL of 300 mM sodium bicarbonate (Sigma, St. Louis, MO) to the dried cDNA sample. Dissolve and mix thoroughly each Alexa dye ARES™ Alexa Fluor® to be used with 5 μL of DMSO (Sigma, St. Louis, MO). Typically Alexa dyes contain two applications in each vial. It is recommended that two sample sets be labeled at the same time. Mix 2.5 μL of dye with the cDNA sample. Incubate in the dark for 1 hour at 25ºC. Determine the pH of 488 Alexa sample before going to purification step using pH paper. It must be lower than 7.0 in order for complete labeled cDNA recovery. Adjust the pH if necessary using 1.0 μL of 2.4 M MOPS at pH 4.0. Mix RT-PCR products with 500 μL Qiagen Binding Buffer and transfer onto filter spin columns, centrifuging for 2 minutes at 16,000 x *g*. Wash out the centrifuge tube with an extra 100 μL of Binding Buffer and centrifuge for <1 minute at 16,000 x *g*. Wash the spin column with 500 μL of 80% ethanol/buffered and centrifuge for 2 minutes at 16,000 x *g* repeat 2 times. The last spin should be a quick pulse to make sure that all of the ethanol is removed. Elute the labeled cDNA 3 times with 20 μL of

nuclease free water at 16,000 x *g* for 1 minute each. Speed vacuum sample in a centrifuge evaporator at 1,000 x *g* and 25ºC (Jouan, Winchester, VA) until approximately 1 µL of volume is left (approximately 30 minutes).

Once the RNA or DNA is labeled with a fluorescent probe the sample can be hybridized to a microarray. The dried fluorescent probes are re-hydrated by adding 5 µL of nuclease free water, and all 4 dyes (488nm, 546nm, 594nm and 647nm) to be used on the microarray are pooled. To the pooled labeled products (pooled volume 20 µL). Add 14 µL hybridization buffer (Amersham, Piscataway, NJ) and 26 µL formamide (Sigma, St. Louis, MO) for a total volume of 60 µL. Incubate at 70ºC for 2 mins, then pipette the sample gently onto the surface of the microarray (see Figure A.8A). Lightly drop 2.5 cm x 6 cm Teflon-edged lifter slip cover slide (Erie Scientific Company, Portsmouth, NH) over the sample (Figure A.8B and A.8C). Incubate the microarray slide in a Corning hybridization chamber (Corning Inc., Corning, NY) at 42ºC for 15 hours. After incubation remove the slide from the chamber and place into a microarray washing chamber (Bass-Pro Shop, Atlanta, GA). Pour 10-20 mL of 2X SSC/ 0.1% SDS (see Appendix I) into the washing chamber with the slide in it and gently swirl solution over slide allowing the cover slip to lift off. Remove cover slip with tweezers. Pour off the wash solution and replace with a fresh solution of 15 mL of 2X SSC/ 0.1% SDS and wash the slide for 5 min. Pour off the liquid and replace with 15 mL of 0.2X SSC and wash for 5 min. Pour off solution again and replace with 15 mL of ultra-pure nuclease free water (Sigma, St. Louis, MO). Centrifuge the microarray slides dry in a Spectrafuge Mini with slide rotor (Labnet International Inc., Woodbridge, NJ). Microarray slides are then ready for scanning as described below.

Once the hybridization and slide washing is complete the microarrays are scanned using a ProScan 3.0 Array Microarray Scanner equipped with four lasers at excitation wavelengths of 488, 546, 594 and 647 nm (Perkin-Elmer, Waltham, MA). Data analysis is carried out using the Scan Array Express Software (Perkin-Elmer, Waltham, MA). The fluorescent intensities generated from the scanner are stored in a Microsoft Excel file which can be copied and pasted into the existing Excel tracking file constructed from a list of positions of PCR products printed on the microarray surface.

RESULTS AND DISCUSSION

Each of the various parts of the microarray process are critical to obtaining good data; it becomes necessary to understand alternative options and prior observed problems/successes at each step. Advances in microarray technology have allowed the industrial market to provide many services related to the microarray approach. Microarray services from companies range from synthesis of oligos, array printing, hybridization of microarray slides, and statistical analysis of data. Nevertheless, direct control and 'hands-on' experience in developing the microarray process is invaluable. This includes generating the DNA elements by PCR, printing the elements, hybridization, and data tracking. Direct involvement also allows one to identify problems and make beneficial improvements. The obvious drawback is the large number of PCR reactions needed to cover one organism. For example *P. furiosus* has a small genome with 2,196 ORFs, which compares to that of *E. coli* which has 4,377 ORFs. The more DNA elements needed the more space on the array is required. Depending on element density and available space on a slide, it should be possible to represent the full genome of an organism on a single slide. Quality control of the PCR elements is critical when constructing a microarray. Those for *P. furiosus* were analyzed by agarose gel electrophoresis to determine that the DNA size corresponded with that predicted for the PCR products. The success rate of the PCR reactions is about 90%, with 10% of the PCR reactions needing to be repeated. This is the average of results from twelve attempts to PCR all ORFs in the *P. furiosus* genome. The length of the PCR product can affect the amount of the labeled probes that binds to the microarray. Also use of PCR products for the microarray versus synthetic oligonucleotides affects the design and efficiency of the microarray. This consideration is dependent on which fluorescent labeling

process is used (i.e. the direct or indirect fluorescent labeling procedure). The direct labeling procedure labels the transcript RNA directly as apposed to the cDNA product of a transcript which is labeled for the indirect method. What is printed on the microarray becomes important in this situation. With PCR products both strands are bound to the slide, insuring binding to either a direct or indirect fluorescently labeled probe. While a synthetic oligonucleotide printed microarray may only reflect the positive or negative coding strand, this may render the array incompatible with a directly fluorescent labeled RNA probe.

Another critical factor in microarray development is the choice of solid support for binding the DNA elements. Typical compositions of solid support materials are glass and plastic with varying surface modifications. Glass slides are used most frequently and were used in the present array as they gave more reproducible results than the plastic slides. Two attributes that can interfere with slide surface chemistry and data collection are autofluorescence and nucleotide binding efficiency. Since this protocol outlines the use of four dyes, covering a broader spectrum of wavelengths, the need to minimize slide autofluorescence is critical. Research suppliers of amino silane slides sometimes undergo a manufacturing change resulting in an increase in 488 nm background although the reason for this is unknown. Each batch of ordered slides must be checked by lot number and tested for auto fluorescence when using the four dye microarray protocol. A batch of slides exhibiting high autofluorescence must be discarded or specifically used with fluorescent probes that would not be affected by the autofluorescence. Currently no method to clean the slides is known to remove autofluorescence. Attempts to wash slides with methanol and ethanol have proven unsuccessful. When an autofluorescence problem arises, the most prudent course is to change to a new supplier of amino silane slides. The second

attribute that microarray slides must possess is binding efficiency of nucleotides, which is dependent upon the surface chemistry. The methods used to bind DNA are nitrocellulose, aldehyde, or amino group modifications (see Appendix H for vendors). While the three surface chemistries were designed to bind DNA, the amino group modified glass slide performed better for the *P. furiosus* microarray by comparison to the other surface modifications. New technologies have been able to create 'pillars' of modified silicon on the surface of microarray slides. These slide are typically referred to as 'high absorbent' slides. Initial testing has proven that these new generation slides would be a welcome alternative to the standard slides once they become more widely available.

Once DNA sequences are covalently bound to the surface of the slide, the sections of the slide that are not covered with DNA elements need to be blocked by non-specific means or else non-specific binding can occur in those locations giving rise to background fluorescent that would interfere with the desired signal. Chemical and salmon sperm DNA blocking has proven effective and result in lowering the background noise (see Appendix B for protocol). Either method has been proven to work well in blocking microarray slides, although the salmon sperm method does not produce hazardous waste and is typically preferred. Blocking the slide must be done before hybridization takes place, or else background noise will be intolerable for data collection.

When isolating total RNA or genomic DNA for microarray hybridization it cannot be stressed enough to make sure all equipment and solutions are RNase and DNase free. RNA and DNA isolation is always carried out using freshly-grown cells (within 12 hours of harvest) and

never from frozen cells. The two methods, phenol/chloroform and silica-membrane spin column, of isolating and purifying RNA or DNA from *P. furiosus* have proven to be reliable, and each method has advantages and disadvantages. The phenol chloroform method yields more total RNA/DNA (~2-3 μg of DAN and ~1-2 μg of RNA from 400 mL at a density of ~1 x 10^8 cells/mL) but produces hazardous waste that requires special disposal. The last step in the phenol/chloroform method for either DNA or RNA isolation requires an extraction with 100% chloroform. This last step must be carried out in order to remove remaining phenol n in the aqueous phase. If not, the RNA or DNA is very difficult to isolate. The benefits of the silica-membrane spin column are that this does not produce hazardous waste. Although a drawback is that the yield from spin column purification of RNA or DNA is low (< 1 μg from 400 mL at a density of ~1 x 10^8 cells/mL). The Corning (Corning, NY) polyethylene caps and polypropylene centrifuge tubes work best for both RNA and DNA procedure as they do not degrade in the presence of phenol/chloroform. If *P. furiosus* cells are grown in the presence of inorganic sulfur, additional washes with suspension buffer are needed before genomic DNA (gDNA) or RNA can be harvested. *P. furiosus* cells grown with S° appear grey and then become more yellow as the cells are washed. Once RNA and DNA has been isolated, purified, and stored either as a isopropanol suspension or aqueous aliquot, samples can be stored at -20°C for up to 2 years. It should be noted that the isolation of genomic DNA from hyperthermophiles other than *P. furiosus* has met with marginal success, for reasons that are not at all clear.

The two methods of labeling RNA and DNA described here involve indirect and direct approaches. Indirect fluorescent labeling was the most efficient method of enzymatically incorporating an attaching fluorophore onto the cDNA for the microarray hybridization. A

disadvantage is that the reverse transcriptase used in this method is not efficient in incorporating the amino allyl labeled uridine nucleotide label into the produced cDNA. Initially, direct fluorescent labeling meant incorporating a covalently connected reporter molecule to a nucleotide which was then inserted into the newly enzymatically generated cDNA. Through technology advancement, the ULYSIS Alexa labeling dye method (Appendix E), enables direct labeling with fluorophores that are attached directly to the isolated transcript RNA and genomic DNA, by direct labeling of the N7 on guanine nucleotides (see Figure A.5). The direct labeling of guanine makes this process interchangeable for both RNA and DNA. It should be noted that the centrifuge columns used for the direct labeling, BIO-RAD Micro Bio-Spin® column, which are very sensitive to the centrifugation speed, as high speeds will damage and compromise the columns ability to purifiy the labeled probe. One consideration must be adressed when using the direct system in conjunction with the microarray. The direct labeling can fluorescently tag transcript RNA which may not be a compliment to the DNA element on the microarry. In this case, a PCR product generated has both positive and negative strands printed on each element and thus avoids this problem. The four dye labeling system has been pioneered by our laboratories, and it has proven to be very effective in gathering multiple comparisons from one microarray slide. At present it is not clear what the upper limit is to the number of different fluorescent probes that can be used on one microarray slide, assuming they have distant excitation and emission properties. Once the labeled probes are generated they can be dried for extended periods of time until being re-hydrated with nuclease free water and for microarray hybridization. Dried labeled probes can be stored for up to 1 year at -20°C.

Hybridization of the fluorescent probes to a microarray is the last step. Once the fluorescently labeled probes are reconstituted with nuclease free water to an appropriate volume, it is important to keep the sample and the microarray slide warm (~35°C). This seems to reduce background 'rings' when applying the probe to the slide surface (see figure A.8C). An alternative technique is to apply the fluorescent probe mixture to the cover slide and to lower the microarray slide, upside down, onto the cover slip. This method is not known to be more efficient or to provide any advantages over other procedures. Bubbles may appear under the cover slip which will cause problems with fluorescent imaging of the elements on the microarray. Removing the cover slide is usually not recommended. Although there is no straightforward way to remove bubbles, tapping, and/or pressing the cover slip sometimes helps. Placing drops of nuclease free water at either side of the cover slip also helps push or draw the bubbles out. Heating the slides can also assist in bubble removal. The commonly used hybridization conditions requires a humid atmosphere with a temperature range of 40-72°C. The different methods of accomplishing the hybridization include a manual chamber on which a microarray slide is placed inside, sealed and incubated at a fixed temperature for a period of time. Other hybridization methods involve more sophistication with automated incubation chambers for temperature, mixing and liquid exchange. Automated and manual hybridization methods have been tested with procedures outlines herein and in my experience the manual hybridization appears to be more reproducible. The automated hybridization has many good features, but maintenance and good product support is needed. Without this, manual hybridization is the best method for dependable and reliable data collection. Once the

microarray slide is dried, the slide can be stored for at least two years in a desiccated, dark place and can still be rescanned several times whenever needed.

Some of the common problems that arise during a microarray experiment are summarized in Table A.1, together with possible solutions. For example, during scanning DNA element morphology may exhibit features known as 'comets' or 'crumbs'. This is a common problem that arises from the microarray slide not being adequately washed after covalently fixing the printed DNA elements to the surface. It is also possible to wash the slides with a dilute solution of surfactant (0.05% v/v Tween-20). The choice of using surfactants must be done with care as some detergents, such as SDS, auto-fluoresce.

Finally, while the microarray technique is based on well established scientific principles, its practice requires an air of an artisan; it is much more art than science. Nevertheless, the techniques and experiences described in this document give reproducible results using RNA or DNA isolated from *P. furiosus*.

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Table A.1: Trouble shooting table for microarray.

Problem	Cause	Solution
Microarray elements are comet shaped	Washing microarray slide after cross linking	Wash slide with more nuclease free water after cross linking step
Microarray elements are faint	Printing error, not enough being transferred via pin quills	Make sure solutions in wells is full enough for printing quill to get adequate
Hybridization resulted in massive non-specific fluorescent binding	1. Hybridization stringency is too low. 2. Hybridization temperature too low. 3. Slide was not blocked 4. Probe sample is contaminated or not of sufficient purity	Increase hybridization temperature Check to make sure slides are properly blocked Repurify RNA or DNA
Microarray missing rows or columns	Printing error which could have resulted from damage pins or water condensation on the pin holder	Check and replace old or damage pins Reprint slides, reduce humidity
Minimum gDNA isolated from *P. furiosus*	1. Cells did not lyse completely 2. Not enough cells used for gDNA isolation.	Try sonicating cells to make sure adequate lysis
Minimum RNA isolated from *P. furiosus*	1. Cells did not lyse completely 2. Not enough cells used for RNA isolation 3. RNAse contamination	Try sonicating cells to make sure adequate lysis Clean all surfaces with RNAse inhibitors
Bio-Rad purification column failure	1. Centrifugation above 1,000 x g causes failure of the column and tends to leak	1. Make absolutely sure centrifugation does not exceed 1,000 x g's.

Figure A.1: Pin arrangement for printing *P. furiosus* microarray slides. The pin holder has a maximum capacity of 8 x 4 pins. Only the first 2 rows of 4 pins are needed for the genome arrangement of *P. furiosus.*

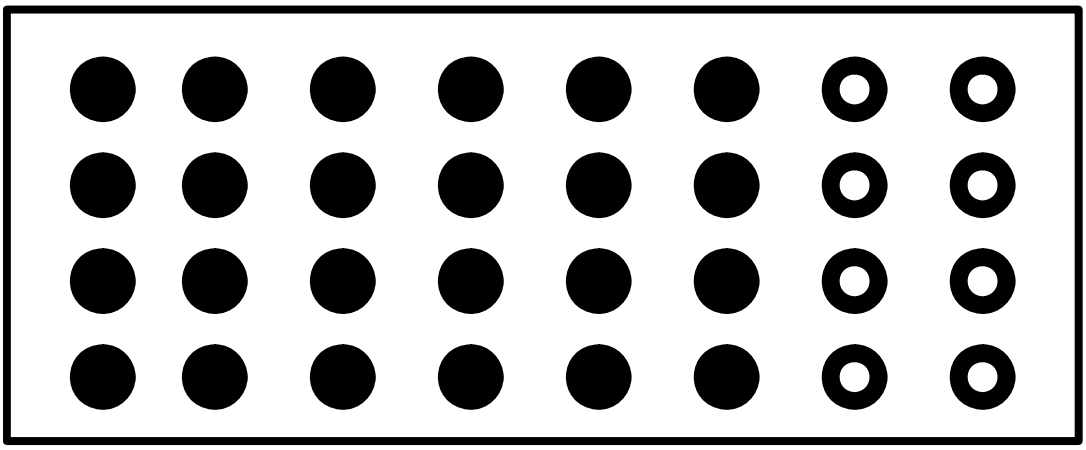


Figure A.2: Graphical representation of the grid alignment of a *P. furiosus* microarray.

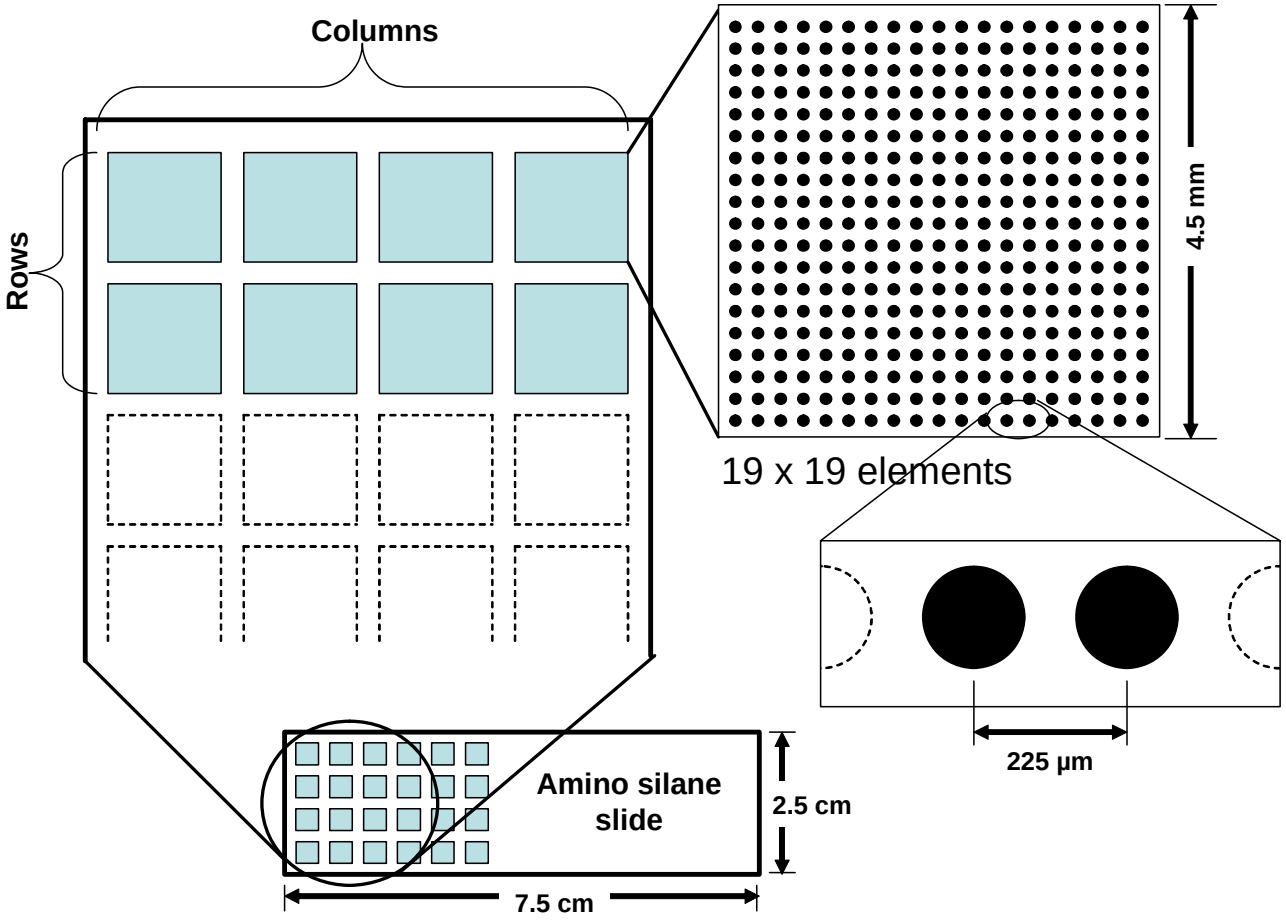


Figure A.3: Interaction of the negative charged backbone of DNA with the positive charged amino silane solid support. This is the first interaction as elements are printed to a amino silane slide.

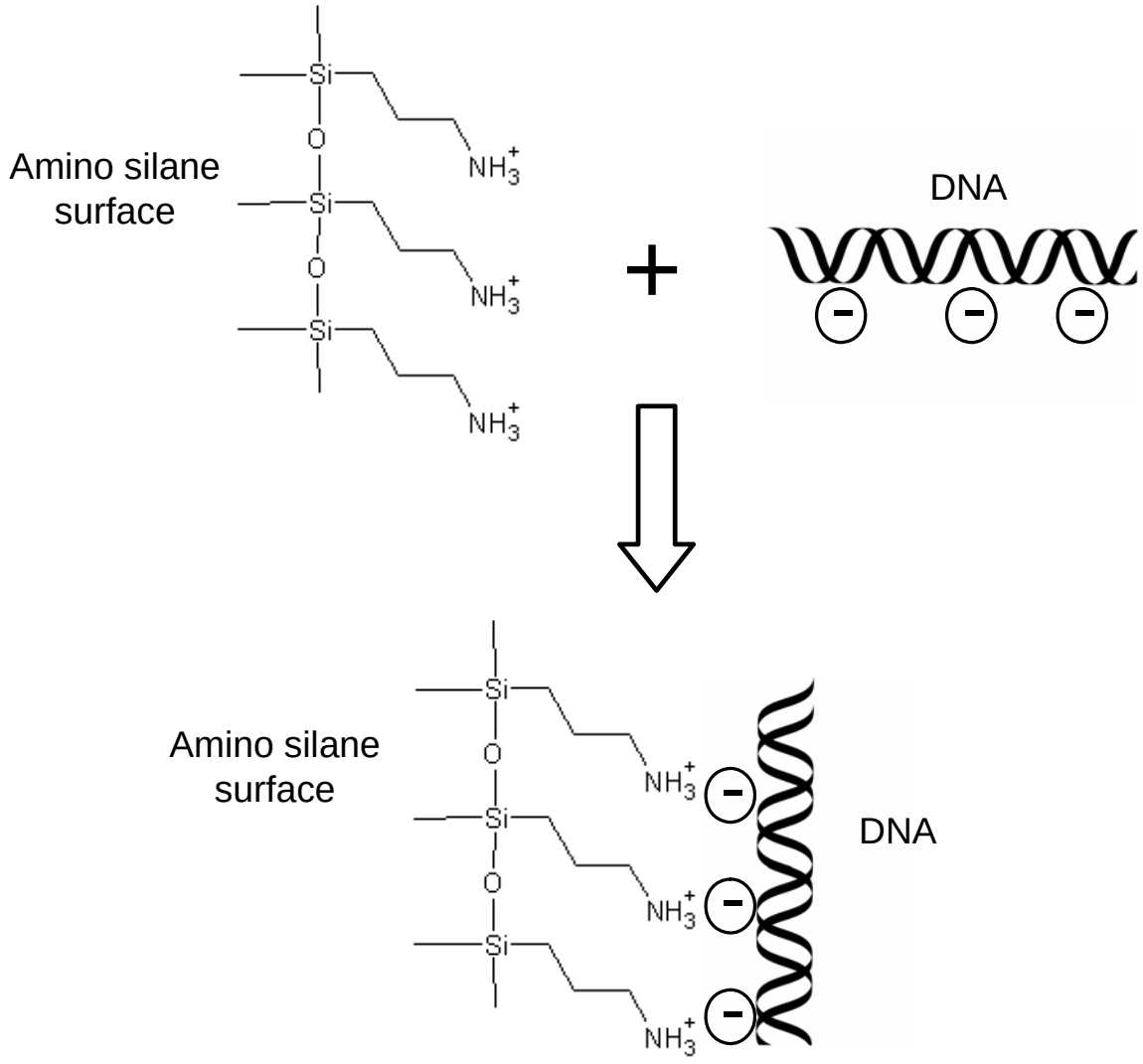


Figure A.4: Chemical blocking of amino silane surface on microarray slide. Primary amine nucleophilically attacks carbonyl on succinic anhydride resulting in a oxygen anion that collapses to break the ether bridge.

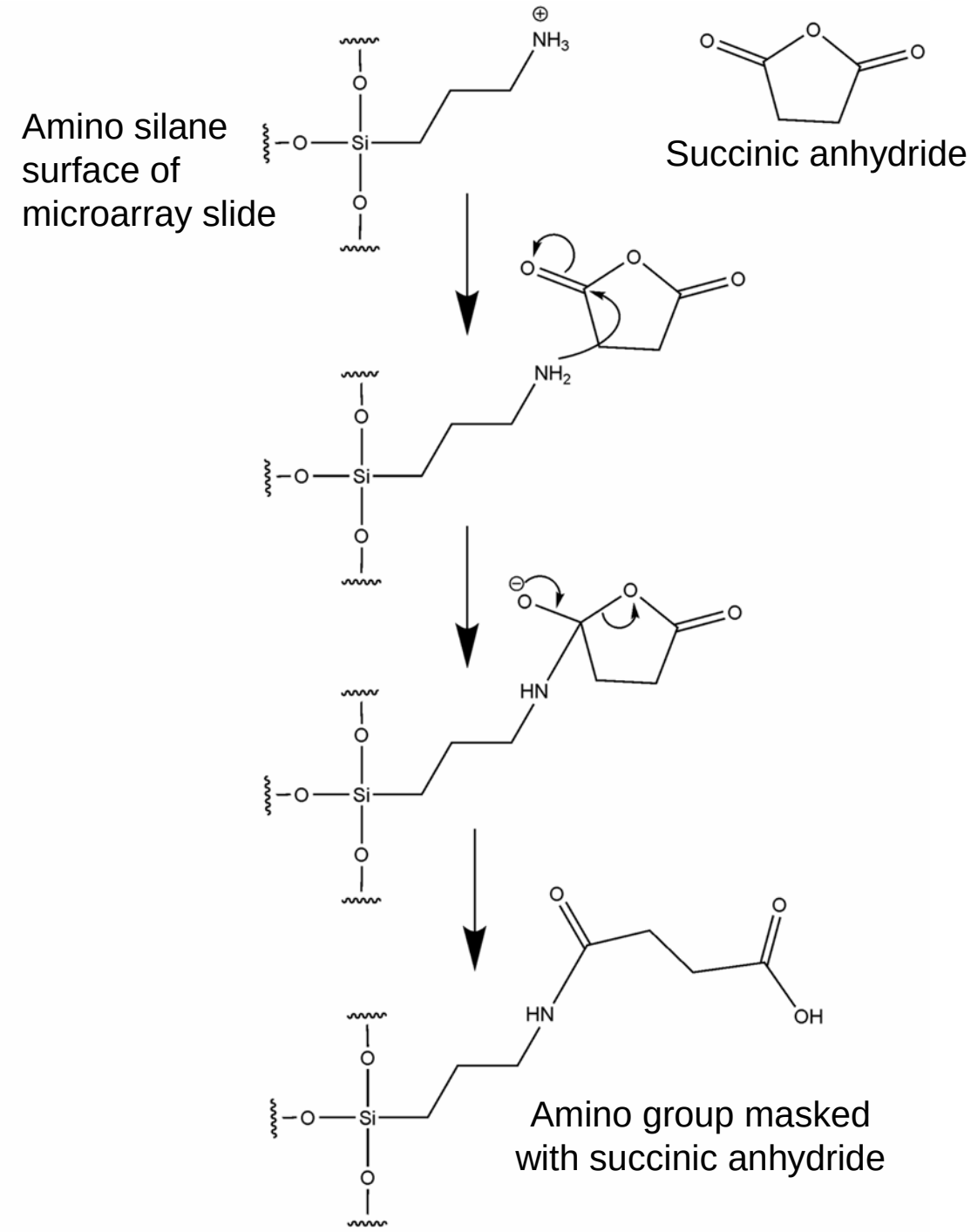


Figure A.5: Direct labeling process using the ULYSIS system. It is presumed the fluorescent label undergoes nucleophilic attack from the 7th nitrogen from guanine.

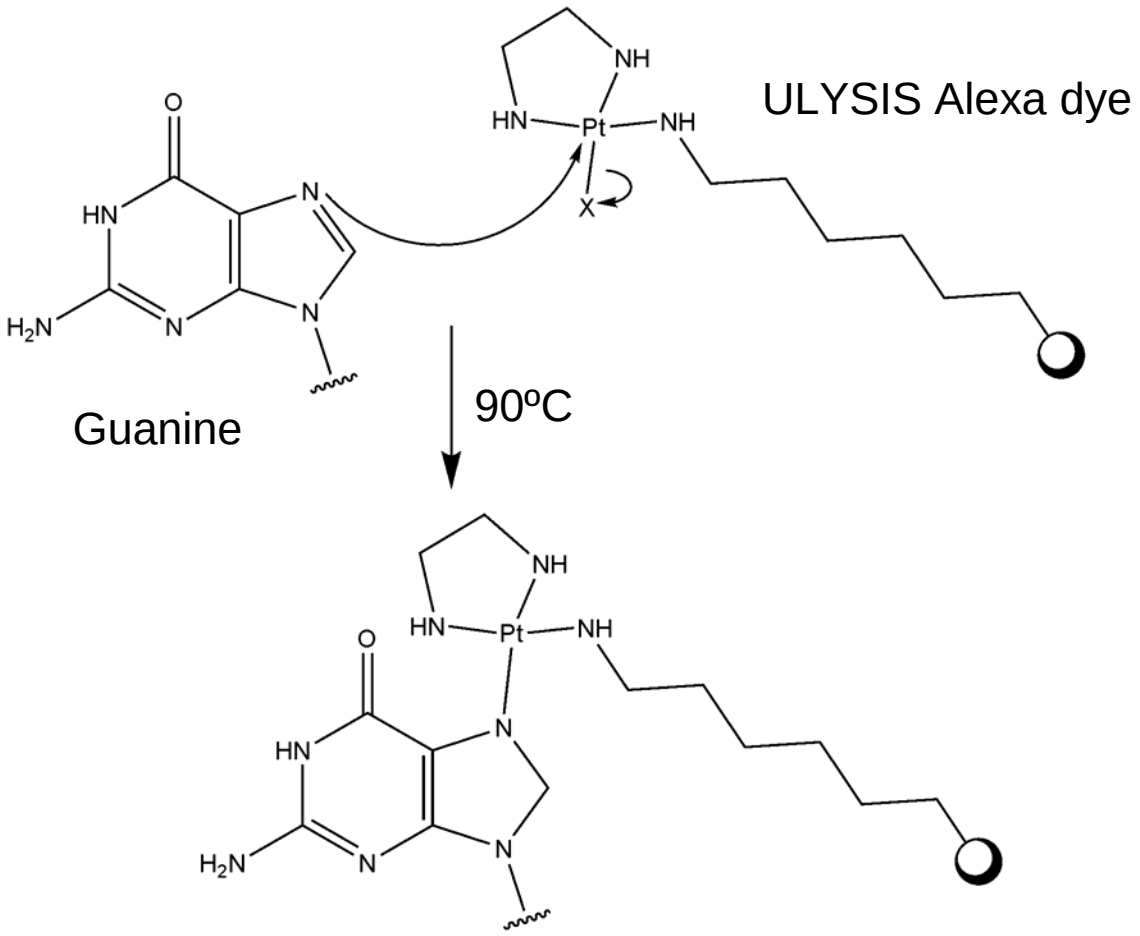


Figure A.6: Covalent attachment of Alexa succinyl ester to cDNA via nucleophilic attack by the incorporated amino allyl Uridine.

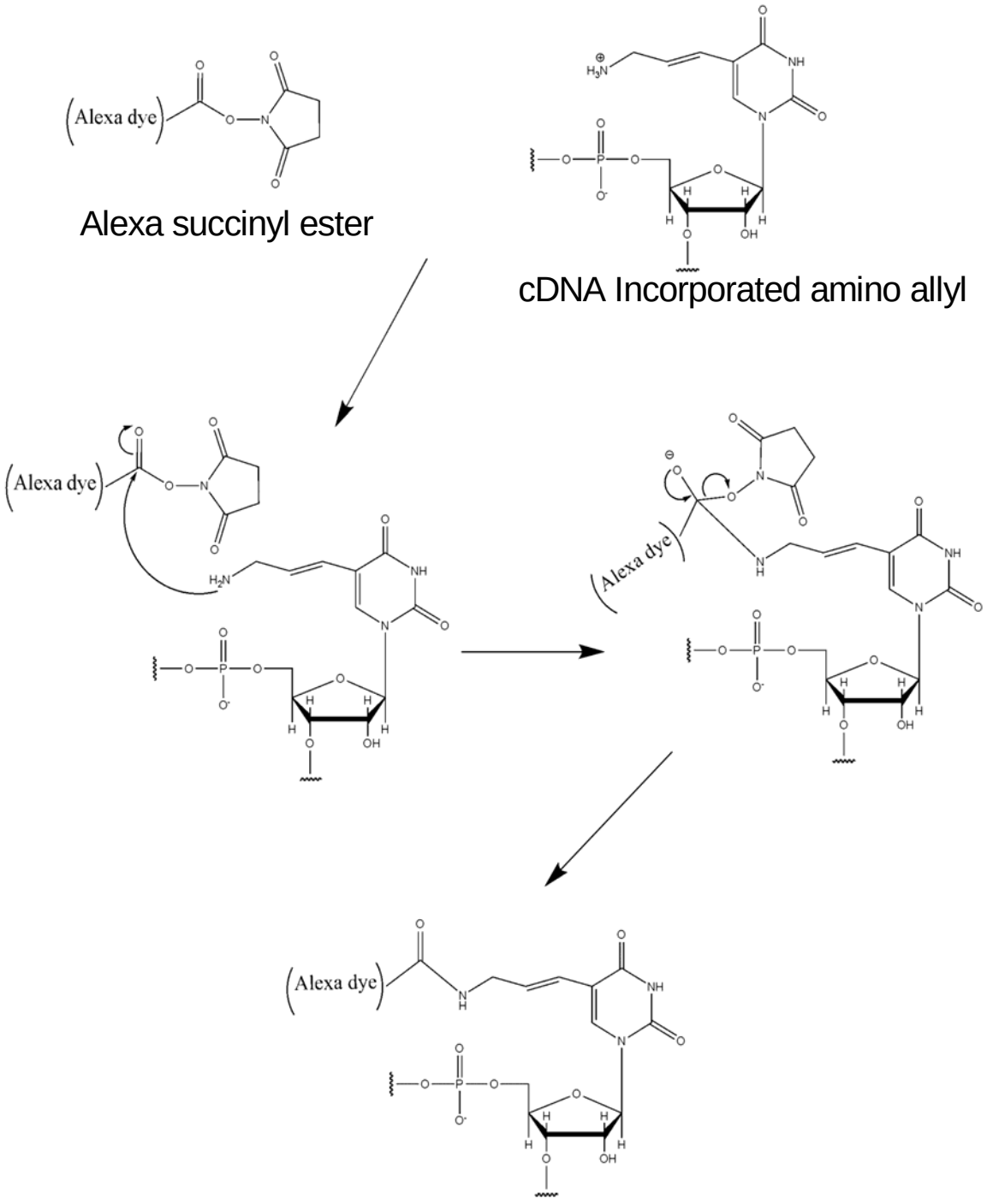


Figure A.7: Variations of the use of microarray. A) traditional transcript reverse transcribing with indirect fluorophor incorporation, B) genomic DNA indirect labeling for genomic comparisons, C) direct fluorescent labeling method which can label either DNA or RNA.

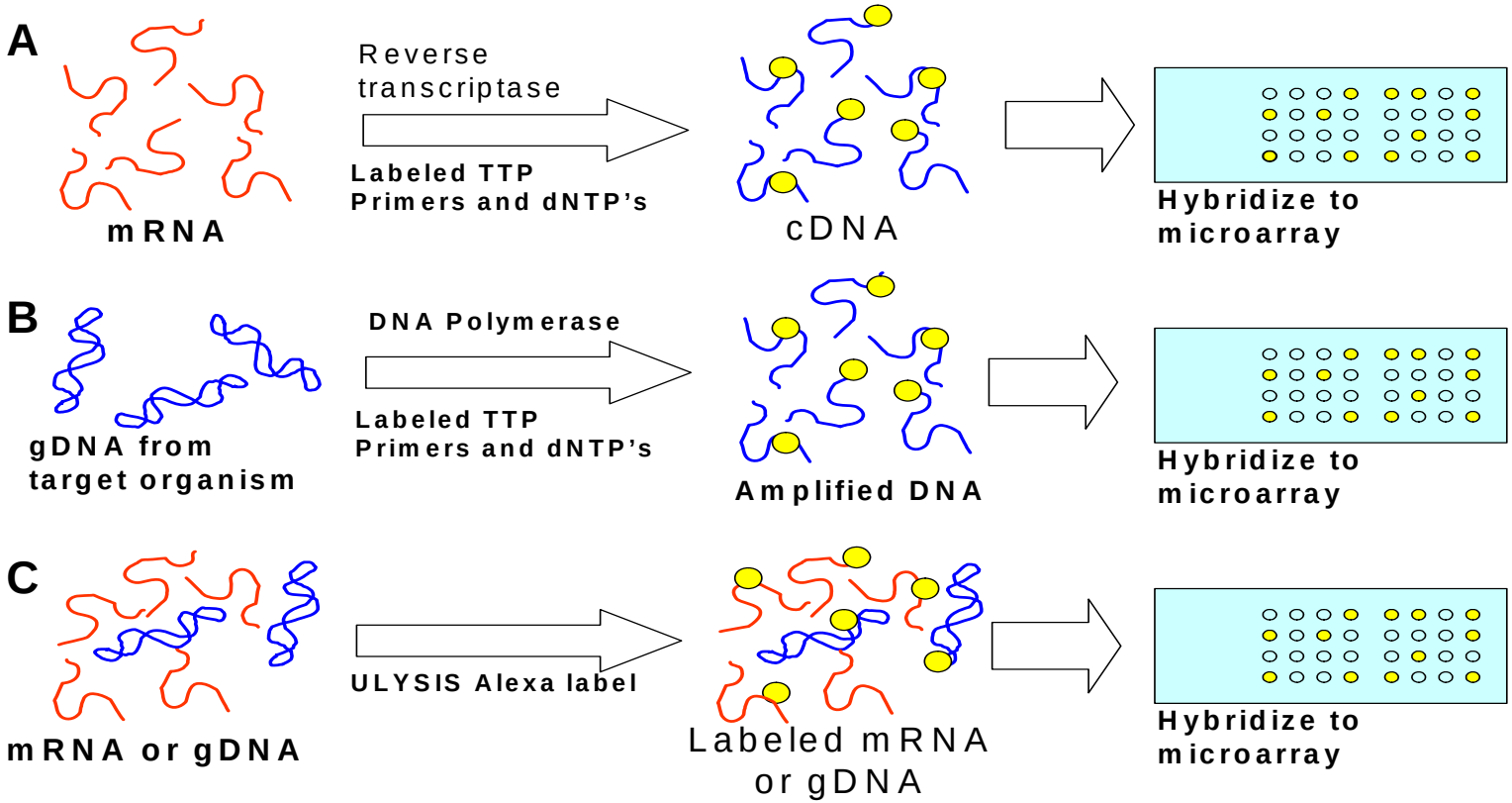
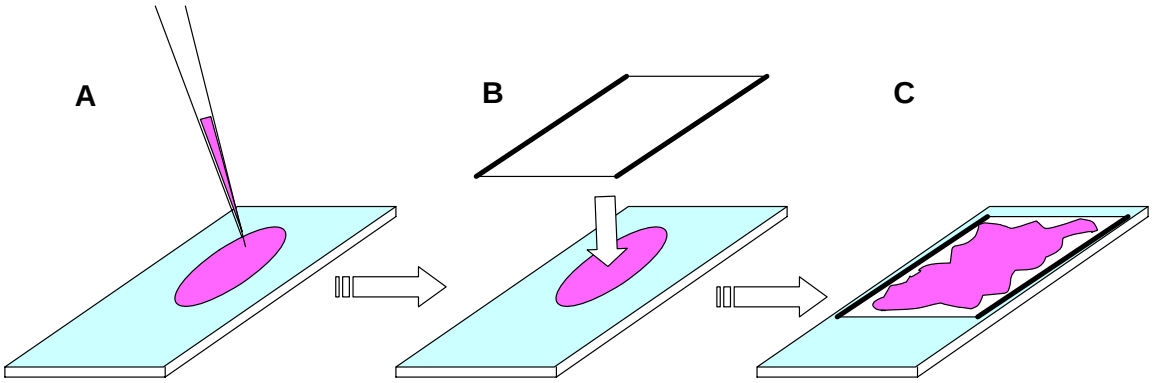


Figure A.8: Loading labeled DNA or RNA onto a microarray slide for hybridization. A) Pipette labeled dye onto microarray slide. B) Gently lower cover slip over microarray slide. C) Making sure bubbles have not been trapped, and place microarray into hybridization chamber.



APPENDIX B. AMINO SILANE SLIDE BLOCKING PROTOCOL

Notes for aminosilane blocking:

1. Avoid touching slide surfaces, when handling hold slides by edges.

2. Use only nuclease free water.

Amino silane slide Blocking

1. Re-hydrate slides by holding over water bath heated to 50 – 65°C for approximately 15 seconds.

2. Place slide immediately on heating block set 80°C for approximately 5 seconds, array side up.

3. Place a set of slides into a UV Crosslinker using 90 mJ (900 μJ x 100 on Stratagene cross linker) of energy, array side up.

4. After UV crosslinking wash slides profusely with nuclease free water.

5. Air dry slides by using a mini centrifuge with a slide adapted rotor. Note: This is a stopping point if need be. Glass slides can be stored at 25°C in a box for a long time (longest has been ½ year).

6. Before continuing with chemically blocking slides prepare a beaker of heated nuclease free water (approx 95°C) and another beaker with room temperature nuclease free water.

7. In a slide incubator chamber dissolve 3.2 g of succinic anhydride (see Appendix H) into 200 mL of 1-methyl-2-pyrolidionone (see Appendix H). Place a spin bar into chamber and vigorously stir solution on a stir plate in a hood.

8. Add 22.2 mL of 0.2 M sodium borate at pH 8.0 to the stirring succinic anhydride solution.

9. Immediately set up a rack of 10 slides and submerge slides 5 times in succinic anhydride solution then leave in solution for 5 minutes. Make sure no slides steeple to an adjacent slide, preventing even flow of solution around slides.

10. Remove rack of slides from organic solution and dunk 3 times in a beaker of 95°C nuclease free water. Leave slides submerged for 2 minutes.

11. Remove slide rack from hot water and place in room temperature water bath.

12. In a separate slide wash chamber fill with 95% ethanol. Individually removed each slide from the room temperature water bath and wash in ethanol chamber for approximately 5 seconds with gentle swirling then air dry with compressed filtered air.

Store slides in a secure, dry, and blocked from sunlight location. Slides are good for at least 1 year.

APPENDIX C. RNA ISOLATION PROTOCOL

Notes for this procedure:

1. Only the Corning polyethylene caps and polypropylene 15 mL centrifuge tubes (see Appendix H) can be used with phenol and chloroform as other plastics will deteriorate and leak.
2. All glassware, plastic containers, solutions must be as RNAse free as possible as this will affect your RNA yields and quality.
3. This procedure uses phenol and chloroform both of these chemicals are hazardous to your health and pregnant women should not handle these components

RNA Isolation: Part 1

1. Collect ~3 liters of *P. furiosus* culture into six 500 mL centrifuge bottles (fill them to about 2-3 cm from rim) in a tub of ice. Centrifuge samples at 4500 x g for 15 minutes cooled to 4˚C.

2. Once finished with first spin, decant supernatant.

3. Make absolutely sure the last drop of medium is removed. Another centrifugation at 4500 x g for 5 minutes cooled to 4˚C maybe required to remove every remnant of medium.

4. Keep samples on ice until phenol/chloroform is added.

5. Using only 1.0 mL of ice cold 3% NaCl combine 2 cell pellets into a 15 mL Corning tube (should result in 3 tubes, each with 1 mL of suspended cells).

6. Add 3.0 mL of Lysis buffer (see appendix I) and 0.35 mL 3.0 M Na-acetate pH 5.0 and 3 mL phenol:chloroform (5:1) acid equilibrated pH 4.7 (see appendix H).

7. Mix well, but do not vortex (solution should turn turbid white).

8. Spin for 20 min, 3500 x *g*; at room temperature in a tabletop centrifuge (since phenol is being use be particularly diligent about cleaning up as everything will begin to smell of phenol).

9. Carefully remove the upper aqueous phase and transfer it to a clean 15 mL falcon tube.

10. To the supernatant add 3.0 mL of phenol:chloroform (5:1) acid equilibrated pH 4.7, mix well and centrifuge for 20 min, 3500 x *g*, at room temperature in the tabletop centrifuge.

11. Carefully remove the upper aqueous phase and transfer it to a clean 15 mL falcon tube.

12. To the supernatant add 3.0 mL of chloroform, mix well and centrifuge for 20 min, 3500 x *g*, at room temperature in the tabletop centrifuge.

13. Make 0.9 mL aliquots in 2.0 mL Eppendorf tube and precipitate the RNA with 1.0 mL isopropanol.

14. Store in -80˚C until needed.

15. When RNA samples are needed for qPCR or microarray purposes follow directions in RNA Isolation Part 2.

RNA Isolation: Part 2

1. Allow samples to equilibrate to 4ºC in an ice bath for approximately 1 hour.

2. Centrifuge RNA samples for 20 min at 16,000 x g, then pipette off supernatant, re-spin the tubes for another 5 min at 16,000 x g and pipette off last drop of liquid.

3. Take up RNA in 100 µL RNase free water and add 500 µL Lysis buffer and 70 µL 3.0 M Na-acetate pH 5.0, mix well.

4. To the supernatant add 600 µL phenol:chloroform (5:1) acid equilibrated pH 4.7 and mix (shake well but do not vortex).

5. Centrifuge samples for 20 min at 16,000 x g at 25°C.

6. Take the water (top) phase and extract this with 600 µL chloroform.

7. Centrifuge samples for 20 min at 16,000 x g at 25°C.

8. Take the water phase (top layer) and add 600 µL isopropanol store sample in freezer, at -20˚C, for 1-2 hours.

9. Centrifuge samples for 20 min at 16,000 x g at 25°C.

10. Pipette off supernatant and wash the pellet with 1.0 mL cold 70% ethanol (Rnase free).

11. Centrifuge samples for 20 min at 16,000 x g at 25°C.

12. Completely remove supernatant, re-spin sample for 2 min at 16,000 x g and pipette off the rest of the supernatant.

13. Air dry the RNA pellet for 5 min. The color of the pellet should be white.

14. Dissolve the RNA pellet in ~100 µL absolute Rnase free water.

15. Measure RNA concentration by diluting 5 µL of concentrated RNA solution into 495 µL of Rnase free water (concentration might be between 0.1 –10 µg/µL). Assay diluted sample by UV 260 λ and 280 λ (RNA OD_{260} of 1 = 40 µg/mL). 260/280 ration should between 1.5 – 2.0 for a pure sample.

16. Once concentrated RNA sample concentration is determined the sample can be diluted or aliquoted then precipitated to for long term storage if necessary.

17. If the sample is to be stored for a long time it is better to precipitate RNA as follows:
    a. Precipitate the aliquot with 0.1 volume of 3.0 M Na-acetate and 3 volumes ethanol then store at -80˚C.

APPENDIX D. DNA ISOLATION PROTOCOL

Notes for this procedure:

1. Make sure all equipment, pipette tips and bottles are nuclease free.
2. Only the Corning polyethylene caps and polypropylene 50 mL centrifuge tubes (see Appendix H) can be used with phenol and chloroform as other plastics will deteriorate and leak.
3. This procedure uses phenol and chloroform both of these chemicals are hazardous to your health and pregnant women should not handle these components

Genomic DNA Isolation

1. Harvest ~800 mL of *P. furiosus* at a concentration of >1.0 x 10^8 cells/mL.

2. Centrifuge cells at 4500 x g for 15 minutes in 4°C.

3. Re-suspend cell pellets in 5-10 mL of Chromosomal DNA isolation Cell Re-suspension Buffer (see appendix I) and transfer to 50 mL conical centrifuge tubes. Only the Corning polyethylene caps and polypropylene 50 mL centrifuge tubes (see Appendix H) can be used with phenol and chloroform as other plastics will deteriorate and leak.

a. Use as little volume as possible, in order to reduce the number of tubes used (Cell pellets may sit on ice overnight if necessary, but not recommended).

4. If cells were grown in the presence of elemental sulfur, this step will separate the whole cells from the sulfur–skip these washes if there is no sulfur. The sulfur will start out grey and then become more yellow as the cells are washed off. Allow the sulfur to settle out and remove the supernatant (The supernatant contains the cells) carefully place into a fresh tube, taking as little sulfur as possible.

    a. Re-suspend the sulfur in another 5-10 mL of Suspension Buffer (see appendix I) and repeat until the sulfur is a bright yellow and no longer dark. At this point, most of the cells will be in the supernatant and most of the sulfur gone. Allow sulfur to settle out of the combined washes and then transfer the supernatant to 50 mL conical centrifuge tubes. Using the Beckman tabletop centrifuge, pellet the cells at 3500 x *g* for 20 minutes 4°C. (Note – Some of the cells have very mushy pellets that are easily lost. These pellets may be stored on ice overnight if absolutely necessary.)

5. Add 50 µL of 20 mg/mL Proteinase K in de-ionized distilled water (DDW) and 125 µL of 10 mg/mL Dnase free Rnase (boil sample to remove Dnase activity).

6. Add 2.0 mL 0.5 M EDTA at pH 8.0, mix and divide into 6 separate 50 mL conical centrifuge tubes (You can use fewer tubes/make the DNA more concentrated, but it will be harder to pipette out later).

7. Incubate at 37°C for 1 hour. Do not shake or vortex sample.

8. Top off each tube to 6 mL with 10 mM Tris-EDTA buffer pH 8 (TE, see Appendix I), mix well by gently swirling (add approx. 4.5 mL 10 mM TE to each tube).

9. Add the following in sequential order to your sample. Do not try to pre-mix these solutions as the salt will cause the detergent to crash out of solution (for all chemicals and solutions see Appendix H and I respectively).

    1.25 mL of 10% SDS

    0.45 mL of 25% Triton X-100

    1.35 mL of 5.0 M NaCl

    1.25 mL of 10% CTAB

    Gently mix by swirling between each addition (Once thoroughly mixed solution turns milky white).

10. Incubate tubes at 65°C for 45 minutes (Note – The tubes may or may not clear, or may even clear, then turn cloudy again, but it seems to make no difference in the final yield.).

11. Add an equal volume ~ 10 mL of 25:24:1 phenol:chloroform:isoamyl alcohol, mix gently but thoroughly. Remember to open tube to allow pressure release.

12. Centrifuge samples in the Beckman table-top centrifuge for 20 minutes at 3500 x *g* set to 4°C.

13. Using a sterile 10 mL glass pipette with pump apparatus to prevent shearing the DNA because of the narrow pipette tip opening. Remove the top aqueous layer containing the DNA to a new 50 mL conical centrifuge tube.

14. Repeat steps #11 - #13 three more times.

    a. The last spin should be at 4500 x g for 15 minutes at 4°C. Make sure no organic are transferred beyond step #14.

15. Add equal volume of chloroform, mix gently, and centrifuge again at 4500 x g for 2 minutes (this should remove the last remaining phenol). Transfer aqueous solution to a conical centrifuge tube.

16. Add 5 M NaCl to reach 10% of total volume (example: 10 mL TE add 1 mL 5 M NaCl).

17. Add 1 – 2 (max) volumes of 100% isopropanol (RT), mix gently and thoroughly. Do not vortex. You should see long strings of chromosomal DNA begin to precipitate out and wind around themselves. If you cannot see the DNA precipitating, this might mean the gDNA is sheared. The sample can be centrifuged and the gDNA collected to be wash as a pellet with 70% ethanol.

18. Melt the tip of a long necked glass Pasteur pipette in the shape of the letter 'J'. Sterilize the hook by flaming briefly with 70% ethanol. Hook each DNA pellet out of the isopropanol and into 15-20 mL of 70% ethanol at 25°C in a 50 mL conical centrifuge tube.

19. Using the Beckman table-top centrifuge pellet DNA at 3500 x *g* set to 4°C for 15 minutes.

20. Decant ethanol and allow pellets to dry inverted briefly ≤5 minutes. Do not dry too much else it will be impossible to re-dissolve. It should still be damp for the next step.

21. Dissolve pellet in a desired volume (~100 µL) of 10 mM TE buffer. Depending on the size of the DNA pellets, adjust the volume of TE buffer. Gently resuspend pellet and allow to dissolve overnight at 4°C.

22. Check optical density, A_{260}/A_{280}, for DNA concentration ($1Abs_{260}$ = 50 µg/mL of dsDNA).

23. Recommended to run ~500 ng of gDNA on a 0.7% (w/v) agarose gel to visualize quality of isolated DNA. For microarray purposes it is okay to have sheared DNA.

24. Aliquot desired amount of DNA into several Eppendorf screw cap tubes. Add 40 µL 3 M Na-Acetate pH 4.5 ($1/10^{th}$ volume) and 1 mL of 100% ethanol. Mix and store at -80^{o}C as an ethanol precipitate for long term storage.

25. To recover gDNA from ethanol long term storage: Centrifuge tube, decant supernatant, wash pellet with 70% ethanol, dry briefly, resuspend in desired volume of 10 mM TE buffer.

APPENDIX E. DIRECT RNA/DNA LABELING PROTOCOL

Direct RNA/DNA labeling notes:

1. Make sure all equipment and reagents are nuclease free.
2. The RNA to be added in this procedure is assumed to be 2 μg/μL.
3. The concentration of genomic DNA to be added in this procedure is assumed to be 1.0 μg/μL.

Direct RNA labeling

1. Mix the following solution using the reagents outlines:

5 μL (0 μL)	RNA (5 μg)/ DNA (1 μg). Dried sample can be used also thus a 0 μL volume, just add 5 μL nuclease free water.
20 μL	Labeling buffer (Component C from ULYSIS kit)
25 μL Total volume	

2. Denature RNA at 65 ºC for 5 minutes (DNA at 95 ºC for 5 minutes) then snap cool on ice bath. Briefly centrifuge to make sure sample is collected on bottom of tube.

3. Mix with the sample the following:

RNA sample (RNA + labeling buffer)

25 µL

4 µL (1 µL) ULYSIS label (previously dissolved in DMF as per Molecular Probes protocol). Note: The ULYSIS Alexa 488 requires 1 µL instead of the 4 µL.

29 µL (26 µL) Total volume

4. Incubate reaction at 90ºC for 10 minutes (15 minutes at 80ºC for gDNA). Stop the reaction by plunging the reaction tube into an ice bath. Centrifuge briefly before purification.

5. Purification of the labeled RNA is accomplished using a spin column. The following steps must be done to prepare the column to be used to purify the labeled RNA.

BIO-RAD Micro Bio-Spin® columns

Invert the column several times to resuspend the gel. Tap tube to remove air bubbles. Break off tip and remove cap allow the column to drain of buffer for 2 minutes into a 2 mL collection tube.

Centrifuge the column at 1,000 x *g* for 2 minutes. Do not centrifuge at higher speeds at any point in this protocol!

Discard the buffer in the collection column. Apply 500 µL nuclease free water and centrifuge at 1,000 x *g* for 1 minute.

Repeat this 3 times.

Discard collection tube and replace with 1.5 mL microfuge tube.

Carefully apply sample to the center of the gel bed.

Centrifuge for 4 minutes at 1,000 x *g*.

Discard column, purified sample is in the 1.5 mL centrifuge tube.

6. Vacuum dry fluorescently labeled samples until all liquid is gone.

7. To each sample add 4.0 µL nuclease free water.

8. Pool the labeled samples together into one tube and add the following to that tube:

16 µL	Labeled sample in tube
13 µL	Hybridization buffer (Amersham, see Appendix H)
26 µL	Formamide (see Appendix H)
55 µL total volume	

9. Incubate at 70ºC for 2 mins.

10. Place sample onto microarray slide and lightly drop cover slip over sample.

11. Incubate microarray slide in Corning chamber at 42ºC for ~15 hours.

12. After incubation wash slide as follows:

5 min	2X SSC/ 0.1% SDS (take cover slip off)
5 min	2X SSC/ 0.1% SDS
5 min	0.2X SSC/ 0.1% SDS
5 min	0.2X SSC
5 min	Nuclease free water

13. Centrifuge dry in minifuge with slide rotor.

Once the slide is dry it can then be scanned using fluorescent scanner.

APPENDIX F. INDIRECT RNA LABELING PROTOCOL

Indirect RNA labeling notes:

1. The RNA to be added in this procedure is assumed to be 2 μg/μL.
2. Make sure all equipment and reagents are nuclease free.

Indirect RNA Labeling

1. In a sterile microfuge tube add 40 μL of 27 OD/mL Random 9-mer Primer (see Appendix H). Dry the primer until no liquid is left.

2. To the dried primer add the following:

Reagent	Volume
RNA (2 μg/μL)	25 μL
Nuclease free water	10 μL
Total volume	35 μL

Mix and spin lightly (approx 1,000 x g)

3. Incubate at 60°C for 15 mins, after incubation immediately inject 1 μL Rnase block (see Appendix H), mix and allow to equilibrate to 25°C. Mix and then spin lightly (approx 1,000 x g).

4. Once equilibrated to 25°C, add the following:

Reagent	Volume
25 mM $MgCl_2$	2.5 μL
10X RT Buffer	10 μL
10 mM dCTP, dGTP, dATP/0.5 mM TTP	10 μL
2 mM aminoallyl dUTP	20.0 μL
Stratascript RT (50 U/mL)	5.0 μL
Total volume	83.5 μL

5. Incubate mixture for 30 mins at 37°C after which add another 3.0 μL of Stratascript RT (50 U/mL) and incubate for another 30 mins at 37°C.

6. Remove the RNA by adding 11 μL of 1 M NaOH and incubate for 8 minutes at 60°C.

7. Neutralize pH with approximately 20 μL of 2.4 M MOPS at pH 4.0 (see Appendix I).

8. Check pH of sample before going to purification step. The pH must be equal or lower than 7.0 in order for binding of labeled cDNA to purification columns.

9. Heat denature sample at 90°C for 1-2 minutes before loading onto spin column.

10. Mix cDNA products with 500 μL Qiagen Binding Buffer (see Appendix H) and filter through spin columns (~1,000 x g).

11. Wash spin column with an extra 100 μL of Binding Buffer (see Appendix H) and spin down (~1,000 x g).

12. Wash spin column 2 times with 500 μL of 80% ethanol/buffered water provided in Qiagen purification kit (see Appendix H). The last spin should be faster (~16,000 x *g*) to make sure all ethanol is removed.

13. Elute labeled cDNA 3 times with 20 μL of nuclease free water into sterile 1.5 mL microfuge tubes (allow the first 20 μL to soak into spin-column for 5 minutes before centrifugation). The desired labeled cDNA is in the water elution.

14. Vacuum dry water sample until damp but not flaky dry.

15. Add 5.0 μL of nuclease free water and 3.0 μL of 300 mM sodium bicarbonate (provided with Molecular Probes Alexa dye, see Appendix H) to the dried cDNA sample.

16. Dissolve and mix each Alexa dye (Alexa 488, 546, 594 and 647, see Appendix H) thoroughly with 5 μL of DMSO.

17. Mix 2.5 μL of dye with amino allyl labeled cDNA sample.

18. Incubate in the dark (wrap in tin foil works well) for 1 hour at 25°C.

19. Mix fluorescently labeled cDNA products with 500 μL Qiagen Binding Buffer and filter through spin columns (~1,000 x g).

20. Wash out centrifuge tube with an extra 100 μL of Binding Buffer and spin down (~1,000 x g).

21. Wash spin column 2 times with 500 μL of 80% ethanol/buffered water provided in Qiagen purification kit. The last spin should be faster (~16,000 x g) to make sure all ethanol is removed.

22. Elute labeled cDNA 3 times with 20 μL of nuclease free water. Speed vacuum sample until dry (not too dry, i.e. don't leave in speed vacuum for over 1 hour).

Sample preparation steps for microarray hybridization:

1. To each sample add 4.0 µL nuclease free water. Making sure each fluorescently labeled cDNA is dissolved in an appropriate volume of nuclease free water.

2. Pool the labeled samples together into one tube and add the following to that tube:

16 µL	Fluorescently labeled cDNA
13 µL	Hybridization buffer (Amersham, see Appendix H)
26 µL	Formamide (see Appendix)
55 µL total volume	

3. Incubate at 70ºC for 2 mins.

4. Place sample onto microarray slide and lightly drop cover slip over sample (Chapter 2, Figure 6).

5. Incubate microarray slide in Corning chamber (see Appendix H) at 42 ºC for ~15 hours.

6. After incubation, remove slides from chamber and place into a shallow wash chamber and wash slide with 20-25 mL as follows:

5 min	2X SSC/ 0.1% SDS (take cover slip off)
5 min	2X SSC/ 0.1% SDS

5 min	0.2X SSC/ 0.1% SDS
5 min	0.2X SSC
5 min	Water

7. Centrifuge microarray slide dry in a minifuge with slide rotor adaptor. Once dry the slide can be scanned using a fluorescent scanner.

APPENDIX G. INDIRECT DNA LABELING PROTOCOL

Indirect DNA labeling notes:

1. Make sure all equipment and reagents are nuclease free.
2. The concentration of genomic DNA to be added in this procedure is assumed to be 1.0 μg/μL.

Indirect Labeling of Genomic DNA

1. In a sterile microfuge tube add 40 μL of 27 OD/mL Random 9-mer Primer (see Appendix H). Dry the primer until no liquid is left.

2. To the dried primer add the following:.

Reagent	Volume
Genomic DNA (1.0 μg/μL)	1.0 μL
Nuclease free water	26 μL
Total volume	27 μL

3. Incubate at 95°C for 5 minutes afterwards plunge gDNA on ice for 5 minutes.

4. After sample has equilibrated to the ice bath add the following:

Reagent	Volume
25 mM $MgCl_2$	1.0
2 mM aminoallyl dUTP	10.0 μL
5X Nucleotide buffer	10.0 μL
Exonuclease-free Klenow (5 U/μL) or	2.0 μL or
Klenow Fragment (40 U/μL)	2.0 μL
Total volume	50 μL

5. Incubate tubes for 120 minutes at 37°C.

6. After the incubation heat denature DNA by incubating at 95°C for 2 minutes.

7. The pH of the sample should be < 7.0 in order for complete binding of allyl labeled cDNA to the purification column.

8. Mix allyl labeled cDNA products with 500 μL Qiagen Binding Buffer and filter through purification column (~1,000 x g).

9. Wash spin column 2 times with 600 μL of 70% wash buffer provided in Qiagen purification kit (~1,000 x g)..

10. Elute labeled cDNA 3 times with 20 μL of nuclease free water into sterile 1.5 mL microfuge tubes (allow the first 20 μL to soak into spin-column for 5 mins before centrifugation, ~1000 x g). The desired labeled cDNA is in the water elution.

11. Vacuum dry allyl labeled cDNA sample until dry.

12. Add 5.0 μL of nuclease free water and 3.0 μL of 300 mM sodium bicarbonate (provided by Molecular Probes Alexa dye kits see Appendix H) to the dried DNA sample.

13. Dissolve and mix thoroughly each Alexa dye to be used with 5 μL of DMSO.

14. Mix 2.5 μL of Alex dye (save other half for another labeling) with prepared allyl labeled cDNA sample.

15. Incubate in the dark (i.e.: wrap in tin foil) for 2 hours at 25°C.

16. Mix DNA products with 500 μL Qiagen Binding Buffer and filter through spin columns(~1,000 x g).

17. Wash spin column 2 times with 600 µL of 70% ethanol/buffered water provided in Qiagen purification kit (~1,000 x g).

18. Elute labeled DNA 2 times with 20 µL of nuclease free water. Allow water to sit on column for 5 mins before centrifugation (~1,000 x g).

19. Vacuum dry sample until no liquid can be seen.

Sample preparation steps for microarray hybridization:

1. To each sample add 4.0 µL nuclease free water. Making sure each fluorescently labeled cDNA is dissolved in an appropriate volume of nuclease free water.

2. Pool the labeled samples together into one tube and add the following to that tube:

16 µL	Fluorescently labeled cDNA
13 µL	Hybridization buffer (Amersham, see Appendix H)
26 µL	Formamide (see Appendix)
55 µL total volume	

3. Incubate at 70ºC for 2 mins.

4. Place sample onto microarray slide and lightly drop cover slip over sample (Chapter 2, Figure 6).

5. Incubate microarray slide in Corning chamber (see Appendix H) at 42 ºC for ~15 hours.

6. After incubation, remove slides from chamber and place into a shallow wash chamber and wash slide with 20-25 mL as follows:

5 min	2X SSC/ 0.1% SDS (take cover slip off)
5 min	2X SSC/ 0.1% SDS
5 min	0.2X SSC/ 0.1% SDS
5 min	0.2X SSC
5 min	Water

7. Centrifuge microarray slide dry in a minifuge with slide rotor adaptor. Once dry the slide can be scanned using a fluorescent scanner.

APPENDIX H. COMPANIES AND CONTACT INFORMATION

Ambion
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Corning
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www.corning.com

Fisher Scientific
Phone: 800-766-7000
FAX: 800-926-1166
www.fishersci.com

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Invitrogen/Molecular Probes
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http://probes.invitrogen.com

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Qiagen Inc.
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www.qiagen.com

Sigma-Aldrich
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FAX: 800-240-4668
www.sigmaaldrich.com

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Phone: 408-744-1331
FAX: 408-744-1711
www.arrayit.com

APPENDIX I. CHEMICALS AND SUPPLIES

Product name	Vendor	Catalogue number	List Price
1-methyl-2-Pyrrolidinone, FW = 99.13, d = 1.03 g/mL,1.0 Liter	Sigma-Aldrich	M-6762	$27.20
5-(3-aminoallyl)-2'-deoxy-uridine 5'-triphosphate	Sigma-Aldrich	A-0410	
ARES™ Alexa Fluor® 488 DNA Labeling Kit	Molecular Probes	A-21665	$226.00
ARES™ Alexa Fluor® 546 DNA Labeling Kit	Molecular Probes	A-21667	$226.00
ARES™ Alexa Fluor® 594 DNA Labeling Kit	Molecular Probes	A-21669	$226.00
ARES™ Alexa Fluor® 647 DNA Labeling Kit	Molecular Probes	A-21676	$226.00
β-mercaptoethanol, 100 mL	Sigma-Aldrich	371300	$14.10
Binding Buffer, 500 mL	Qiagen	19066	$45.00
Chloroform, 4 L, HPLC grade	Fisher	C606-4	$39.50
Corning 15 mL centrifuge tubes, sterile, package of 50	Corning	725700	$11.23
Corning 50 mL centrifuge tubes, sterile, case of 500 tubes	Corning	725815	$122.75
Corning microarray chambers, 5/case	Corning	2551	$296.74
dNTPs,100 mM	Sigma-Aldrich	DNTP-100	$215.65
Ethanol (Absolute), 6 X 500 mL	Sigma-Aldrich	E 7023	$132.00

Exonuclease Klenow Polymerase,125 U	Stratagene	600069	$96.00
Genorama® microarray slides, 25 slides	Asper Biotech	SA-1	$173.75
Guanidine thiocyanate, 500 g	Sigma-Aldrich	G 9277	$187.00
Hexadecyltrimethylammonium Bromide, Sigma Ultra, 100 g	Sigma-Aldrich	H9151	$64.80
Isopropanol, 4 L, HPLC grade	Fisher	A451-4	$78.49
Microarray Hybridization buffer	Amersham	RPK0325	$115.00
Micro Bio-Spin 30 Columns, RNAse free, 100 units	Bio-Rad	732-6251	$273.00
MicroMax™ glass slides Super Chip™ I	Perkin-Elmer	MPS696	$220.00
N-lauroylsarcosine sodium salt, 100 g	Sigma-Aldrich	L 9150	$25.20
Phenol: Chloroform: Isoamyl alcohol, 25:24:1, pH 8.0, 400 mL	Sigma-Aldrich	P2069	$107.40
Phenol : Chloroform 5:1, acid equilibrated pH 4.7, 400 mL	Sigma-Aldrich	P1944	$91.50
Proteinase K, 100 mg	Sigma-Aldrich	P6556	$71.10
Promega PureYeild™ RNA Midiprep System, 50 prerps	Promega	Z3741	$450.00
QIAquick PCR Purification Kit (250)	Qiagen	28106	$350.00
QIAquick 96 PCR Purification Kit (24) For purification of 24 x 96 PCR reactions: 24 QIAquick 96 Plates, Buffers, Collection Microtubes (1.2 ml) and Caps	Quiagen	28183	$2,408.00
Random 9-mer Primer, 350 µL	Stratagene	300309	$153.00
RNase Block, Recombinant ribonuclease inhibitor	Stratagene	300152	$345.00
RNAqueous-4 PCR	Ambion	1914	$140.00

RT-Buffer,10X	Stratagene	600085	
Sodium Borate 10-hydrate, FW = 381.4, 500 g	Sigma-Aldrich	S-9640	$15.70
Sodium acetate, anhydrous ultrapure, 500 g	Sigma-Aldrich	S 7545	$34.70
Stealth Micro Spotting pins	Telechem International Inc.	SMP3	$300.00
StrataScript™ Reverse Transcriptase, 10,000 U	Stratagene	600085	$155.00
Succinic anhydride, FW = 100.1, 500 g	Sigma-Aldrich	S-7626	$13.00
ULYSIS® Alexa Fluor 488, nucleic acid labeling kit	Invitrogen/Molecular Porbes	U21650	$277.00
ULYSIS® Alexa Fluor 546, nucleic acid labeling kit	Invitrogen/Molecular Porbes	U21652	$277.00
ULYSIS® Alexa Fluor 594, nucleic acid labeling kit	Invitrogen/Molecular Porbes	U21654	$277.00
ULYSIS® Alexa Fluor 647, nucleic acid labeling kit	Invitrogen/Molecular Porbes	U21660	$277.00

APPENDIX J. BUFFERS AND SOLUTION PREPARATIONS

20X Sodium chloride/sodium citrate buffer (SCC) (1 Liter)

175.32 g Sodium chloride (final conc. 3 M)

88.23 g Sodium citrate (final conc. 0.3 M)

Dissolve into 1000 mL water, adjust pH to 7 (using 1 M HCl or 1M NaOH) and filter sterilize.

Chromosomal DNA isolation Cell Re-suspension Buffer (250 mL)

62.5 g of sucrose (final conc. 25% w/v)

1.5 g of Tris at pH 7.8, FW = 121.14 (final conc. 50 mM)

3.7 g of EDTA, FW = 372.74 (final conc. 40 mM)

Dissolve in 250 mL water and filter sterilize.

10 mM Tris EDTA (TE) Buffer

10 mM Tris at pH 8.0 (FW = 121.14)

1 mM EDTA (FW = 380.2)

Autoclave solution

10% w/v SDS (50 mL)

5 g of sodium dodecyl sulfate

Fill to 50 mL with DDW.

25% Triton-100 (50 mL)

12.5 mL of Triton X-100

Fill to 50 mL with DDW

5.0 M NaCl (200 mL)

58.44 g of NaCl, FW= 58.44

Fill to 200 mL with DDW

10% CTAB (100 mL)

10 g of Hexadecyltrimethyl ammonium bromide (CTAB)

Dissolve in 100 mL 0.7 M NaCl.

Pre-hybridization buffer

100 mL 20x SSC @ pH 7

10 mL 10% SDS

10 mL 10 mg/mL Salmon Sperm DNA

880 mL de-ionized distilled water

(Prepare in a 1 L bottle and sonicate at 100ºC)

Isopropanol and Chloroform

Use freshly autoclaved bottles for easy handling and aliquot amounts needed of either chemical (see Appendix H for suppliers). Use caution when pouring these chemicals.

Cell Lysis buffer (50 mL)

23.6 g Guanidine thiocyanate (FW = 118.2) (4.0 M)

415 mg 0.83% N-lauryl sarcosine (FW = 293.4)

Dissolve in DDW (deionized distilled water) then add 0.5 mL of 3.0 M Na-acetate pH 5.0

Adjust to 50 ml, make sure the pH is about 5 and the solution is colorless (use 1 M HCl or 1M NaOH as needed)

350 µL of 100 mM β-mercaptoethanol (add after autoclaving!) (d = 1.114 g/mL, FW = 78.13).

3.0 M Na-acetate @ pH 5.0 (50 mL)

12.3 g Na-acetate (FW = 82.03) (3.0 M)

Adjust pH to 5.0 using concentrated glacial acetic acid (Sigma). Autoclave 50 mL batch of sodium acetate and several 2 or 3 dram vials with lids to aliquot into after solution has cooled from autoclaving.

70% Ethanol (RNase free)

Use a freshly autoclaved bottle and make a 100 mL of the 70% ethanol solution (70 mL ethanol and 30 mL dionized distilled water).

3% NaCl Solution (100 mL)

3.0 g of NaCl

Dissolve in 100 mL DDW.

5X -Exo Klenow Polymerase buffer (10 mL pure water)

41.2 mg	Tris-base @ pH 7.5 (FW: 121.1) final conc. 34 mM
10.2 mg	$MgCl_2$ (Fw 203.0) final conc. 5 mM
65 mg	Dithiothreitol final conc. w/v 0.65%
10 µL	100 mM of dCTP, dGTP and dATP final conc. 100 µM
2 µL	100 mM TTP final conc. 20 µM

Store in aliquots at -80°C.