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Deoxyribonuclease Activity from Helical Bacteria

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Dedication:

To my father and mother...

To my husband, brother, and family...

To my son Ibrahim...

To my doctors...

Areej Sami Altaweel

Declaration

I certify that this thesis submitted for the degree of Master in Medical Laboratory Sciences/ Microbiology and Immunology track, is the result of my own research, except where otherwise acknowledge, and that this study has not been submitted for a higher degree to any other university or institution.

Signed

Areej Sami Altaweel

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Abstract

The activity of nuclease enzyme in helical bacteria such as *Campylobacter jejuni*, *Helicobacter pylori*, and *Alphaproteobacteria* "QUBC 70" has been detected. This activity interferes with DNA preparation, isolation, storage, and DNA-based reactions including PCR and sequencing. These bacteria are fastidious; slow growing, Gram negative helical bacteria. The high rate of infections with *H. pylori* (50% worldwide) and 85% among Palestinians (and most likely the same rate with *Campylobacter jejuni*), dictates that a reliable molecular method of detection of these bacteria from stool, water, food, or other samples must be established. DNA based detection has been hampered by such putative nucleases. This work focused on expanding our understanding of the characteristics and properties of these nucleases and to explore the conditions for having stable DNA preparation from these bacteria without interference of nucleases.

Bacterial cultures from stool were used to collect bacteria for lysis. Lysates were prepared by different methods; SDS lysis, SDS and boiling, sonication, lysozyme and water. SDS-lysis was selected as the method of choice. Water saturated with Ammonium sulfate (AS) was used to fractionate proteins from cleared lysate.

This work illustrates the presence of DNase activity in bacterial lysate prepared by lysing bacterial cells in the presence of SDS followed by boiling, indicating the putative nuclease to be SDS-heat stable. When lysis was performed with lysozyme or sonication without SDS, the putative nuclease appeared to be reduced probably due to the proteases and/or due to nuclease association with the cell envelope. The addition of saturated AS (AS; 0.6 1 v/v at 25˚C) to *C. jejuni* lysate precipitated the nuclease. Ammonium sulfate was not efficient in salting-out the nuclease activity when applied at <0.6 volumes of *Campylobacter* lysate. When applied to a different bacterial lysate (*Alphaproteobacteria* QUBC 70), AS precipitated the nuclease activity at ~300%. The DNase activity was assayed by mixing exogenous λ-DNA (lambda bacteriophage) with target preparation and incubation at 36˚C or 45˚C for different times up to 48 hours.

It can be concluded that repeatedly, the DNase activity was found in cell extracts of both *C. jejuni* and "QUBC 70". Ammonium sulfate DNase precipitation profile for *C. jejuni* was different and distinguishable from that of the *Alphaproteobacteria* QUBC 70.

Campylobacter nuclease was active at 36 ^{o}C and 45˚C but poorly at 50˚C in the presence of > 0.01% SDS.

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List of abbreviations

2-ME	2-Mercaptoehanol
ATCC	American type culture
C. jejuni	*Campylobacter jejuni*
DNase	Deoxyribonuclease
dsDNA	Double-stranded DNA
E. coli	*Escherichia coli*
H. felis	*Helicobacter felis*
PCR	Polymerase chain reaction
Prk	Protease K
QUSB 70	Quds universal 70
S. aureus	*Staphylococcus aureus*
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TAE	Tris acetate EDTA
TBO	Toludine blue O
TE	Tris buffer
V/V	Volume/volume
VBNC	Viable but non culturable

Chapter I

Introduction

1.1 Helical Bacteria

Bacteria such as *Helicobacter pylori* and *Campylobacter jejuni* are medically important pathogens that require monitoring and continuous assessment of their presence in the environment, in healthy, carriers, and sick individuals and animals. In general, they can inhabit various niches in the environment. *Helicobacter pylori* inhabit areas of the stomach and duodenum. It causes a chronic low level inflammation of the stomach lining, gastritis, and is strongly linked to the development of duodenal ulcers, gastric ulcers, and has been classified as Class I carcinogen (Bargouthi, 2009). *Helicobacter pylori* was recognized as Campylobacter-like organism until it was given the current species status. Since then, tens of Helicobacter species have been discovered and are known to be strongly host specific; *H. felis* is the species found in most cats while *H. suis* is found in swine species. Few *Helicobacter* spp. have been reported to cause human diseases especially *Helicobacter hepaticus* (Bargouthi, 2009).

Common features among these species include shape (spiral or helical) and that they inhabit the intestines of the target host. In the laboratory, they are slow growers; require low oxygen tension (1-5%) and serum, blood, or a substitute (Bargouthi, 2009). Another important character that is controversial; is the ability of these species and others

(including *Legionella*, *Vibrio*, *Salmonella*, *Escherichia*, and *Shigella* spp., other spiral bacteria that show coccoid forms include *Desulfovibrio*, *Campylobacter*, *Aquaspirillum*, *Oceanospirillum*, and *Spirillum*) to morphologically transform to a coccoid form also known as viable but non-culturable (VBNC) form. Coccoid and VBNC forms may be critical stages that allow undetectable transfer and survival of the bacterium, this view is supported by the ubiquitous distribution of such bacteria. Resuscitation of 30-day old (VBNC) *Campylobacter jejuni* through an embryonated hen-egg was reported to allow recovery of the bacterium from a large proportion of the inoculated eggs**.** Other investigators view these forms as dead bacteria (Bargouthi, 2009).

Detection of *Helicobacter*, *Campylobacter*, and other fastidious pathogens and environmental bacteria becomes a serious problem due to the culture-evading forms and the difficulty of extracting stable DNA from them. The original observation is that crude DNA extracted from cultured *Helicobacter* or *Campylobacter* is highly unstable unlike those of *Escherichia coli* and *Bacillus* spp. (Barghouthi, 2011). Taken together, the following factors; VBNC forms, low infectious doses, slow growth, unstable DNA in crude cell lysate, and the vague issue of DNA extraction from coccoid forms, the detection and identification of such bacteria may be inefficient and subject to failure (Bargouthi, 2009; Nogva et al., 2000).

1.2. Literature Review

1.2.1 Nucleases

Under well contained laboratory conditions, DNA instability is essentially due to the presence of free metals and/or dexoribonucleases since the double helix is highly stable. Watson and Crick description of the double helix of the DNA molecule opened the doors to a new area in biological understanding and research. In the late 1960s, scientists Stuart Linn and Werner Arber isolated two types of enzymes responsible for phage growth restriction in *Escherichia coli* bacteria. One of these enzymes added a methyl group to the DNA, generating methylated DNA, while the other cleaved unmethylated DNA at a wide variety of locations (*dam* and *dcm*) along the length of the molecule. In 1968 Smith, Wilcox, and Kelley, working at Johns Hopkins University, isolated and characterized the first restriction nuclease whose function depends on a specific DNA nucleotide sequence.

Working with *Haemophilus influenzae* bacteria, this group isolated an enzyme, called Hind III, that cuts DNA molecules at a particular point within a specific sequence of six base pairs as reported by (Roszczyk and Goodgal, 1975).

Nucleases are enzymes that break phosphodiester bonds of DNA or RNA polymers. Nucleases, which belong to the class of enzymes called hydrolyses, are usually specific in action; some enzymes having a general action (such as phosphoesterases, which hydrolyze phosphoric acid esters) can be called nucleases because nucleic acids are susceptible to their action. Enzymes that cleave the phosphodiester bonds of DNA are called deoxyribonucleases, and enzymes that cleave the phosphodiester bonds of RNA are called ribonucleases. To cut the DNA, a restriction enzyme is a nuclease that splits only those DNA molecules in which they recognize particular subunits. Some split the target DNA molecule at random - makes two incisions, once through each sugar-phosphate backbone of the DNA double helix. These enzymes have different roles in the synthesis of DNA molecules, the attachment of two or more DNA molecules to one another, and the breaking of DNA molecules into fragments. Importantly, these enzymes allow bacteria to take up DNA from its environment and become genetically transformed with these DNA fragments, making it possible to modify and their genomes, equally important, to manipulate the function of the genes located on these new genomes (den Bakker et al., 2008; Maughan and Redfield, 2009) .

Horizontal genetic exchange was shown to take place in *C. jejuni* and to be responsible for pathogen biodiversity as shown in experimental infection of chickens (de Boer et al., 2002).

In 1955 Arthur Kornberg and colleagues isolated DNA polymerase (Kornberg et al., 1955; Weiss et al., 1968a; Weiss et al., 1968b; Weiss et al., 1968c). Smith, Wilcox, and Kelley as reported by (Roszczyk and Goodgal, 1975) isolated and characterized the first sequence specific restriction nuclease in 1968. These enzymes, respectively, play different roles in the synthesis of DNA molecules, the attachment of two or more DNA molecules to one another, and the breaking of DNA molecules into fragments. Importantly, these enzymes make it possible to create entirely new kinds of DNA molecules and, equally important, to manipulate the function of the genes located on these new molecules. Nucleases are

divided into two classes: exonucleases and endonucleases, based on position of the cleaved bond within DNA or RNA polymers.

The exonucleases are involved in trimming the end of RNA and DNA polymers, cleaving the last phosphodiester bond in a chain. This cleavage results in the removal of a single nucleotide from the polymer. If the enzyme removes nucleotides from the 3'- or 5'- end it is referred to as exonuclease. The specificities exhibited by nucleases reflect the wide biological functions for these enzymes. Prokaryotes are like eukaryotes, they all have three types of nucleases depending on the normal turnover of mRNA. In eukaryotes, a 5'→3' exonuclease, which is a decapping protein that is required to remove the protective cap from the 5'-end of eukaryotic mRNA.The3'→5'exonuclease, is a poly(A)-specific 3'→ 5' exonuclease. The third, is an endoribonuclease (Wang and Kiledjian, 2000).

As in eukaryotes, archaebacteria degrade RNA utilizing a protein complex (11-16 proteins which include exonucleases, helicases, and RNA binding proteins (Tahirov et al., 2009). Since the discovery of DNA exonuclease I in *E. coli*, several other DNA exonucleases have been discovered (exonucleas II-VIII) (Lehman and Nussbaum, 1964; Mukherjee et al., 2002)

The second group of endonucleases cleave phosphodiester bond of DNA or RNA at a position other than the ends of polymers, producing fragment of DNA or RNA. A restriction endonuclease functions by scanning the length of a DNA molecule, once it encounters its particular specific recognition sequence; it binds to the DNA molecule and makes one cut in each of the two sugar-phosphate backbones of the double helix. The positions of these two cuts, their relation to each other and to the recognition sequence itself, are determined by the identity of the restriction endonuclease used to cleave the molecule in the first place. Different endonucleases yield different sets of cuts, but one endonuclease will always cut a particular base sequence the same way, no matter what DNA molecule it is acting on. Once the cuts have been made, the DNA molecule will break into fragments and the resulting fragments are held together only by the relatively weak hydrogen bonds that hold the complementary bases to each other. The weakness of these bonds allows the DNA fragments to separate from each other. Each resulting fragment has a protruding 5'- end composed of unpaired bases. Other enzymes create cuts in the DNA backbone which result in protruding 3'- ends. Protruding ends both 3'- and 5'-

are sometimes called sticky ends because they tend to bond with complementary sequences of bases, others make blunt end cuts with the two DNA strands having flushed ends. Ligase enzyme is then used to join the phosphate backbones of the two molecules. The cellular origin, or even the species origin, of the sticky ends does not affect their stickiness. Any pair of complementary sequences will tend to bond; even if one of the sequences comes from a length of human DNA, and the other comes from a length of bacterial DNA. But Restriction endonucleases are categorized into three general groups: Types I- were the first to be identified and characterized from two different strains of *E. coli* and these enzymes cut at a site that differs, and is some distance at least 1000 bp away from their recognition site; type-II are composed of only one subunit, their recognition sites are usually undivided palindromic and 4-8 nucleotides in length, they recognize and cleave DNA at the same site, and they do not use ATP for their activity, they usually require only Mg^{2+} as a cofactor; type III cut DNA about 20-30 base pairs after the recognition site, these enzymes contain more than one subunit and require ATP cofactors for DNA methylation and restriction. Classification is based on their composition and enzyme cofactor requirements, the nature of their target sequence, and the position of their DNA cleavage site relative to the target sequence as shown for the restriction endonuclease HpyAV from *H. pylori* (Chan et al., 2010).

Gaasbeek et al. (2010) have shown that differences in nuclease activity exist between competent and noncompetent *C. jejuni*; a subset of strains has a periplasmic DNase (encoded by *dns* gene) which inhibits natural transformation in *C. jejuni*.

1.2.2 Deoxyribonucleases (DNases) from *Helicobacter* and *Campylobacter*

It appears that deoxyribonucleases with several functions and distributions among bacteria are far from being settled anytime in the near future. Taking into consideration the number of nucleases per species, the number of species, the number of researchers, and the required time for each successful investigation limit our knowledge of this class of enzymes which is only second to DNA importance. The absence of comprehensive reports on DNases makes any efforts in this area of research an important contribution. *H. pylori* and other *H. spp* are associated with a multitude of human and animal diseases. The genus includes more than 24 formally recognized species and more than 35 novel species

awaiting formal nomenclature and will most likely expand to encompass additional species (Bargouthi, 2009).

1.2.2.1 *Campylobacter jejuni*

C. jejuni is a leading cause of zoontic diarrhea, food-and water-borne gastro-enteritis. It is widely distributed with many animal reservoirs. Fecal-oral transmission that may survive in a dormant form (VBNC) (Nogva et al., 2000).

Campylobacter jejuni, one of the subunits of toxin CdtB has a type I deoxyribonucleases activity (Lara-Tejero and Galan, 2000). *Campylobacter jejuni* is naturally competent for DNA uptake. In addition to the *dns* gene coding for DNase*;* two other genes, that are DNA/RNA nonspecific endonucleases, are also encoded by CJE0566 and CJE1441 of strain RM1221. These genes are located on *C. jejuni* integrated elements 2 and 4. *Campylobacter jejuni* DNases belong to the *Serratia* nuclease family. Researchers concluded that the nucleases were acquired from a bacteriophage. The nucleases inhibit the competency of *C. jejuni* for transformation (Gaasbeek et al., 2010).

1.2.2.2 Helicobacter nucleases

Helicobacter pylori are naturally competent for transformation; evidence for the active role of the membrane associated NucT nuclease in genetic transformation, i.e. uptake of DNA from the environment was detected. NucT is a cation-independent thermostable nuclease; it preferentially cleaves single-stranded DNA. The gene/open-reading frame hp0323 codes for a nuclease of *H. pylori*. A strong nuclease activity can be detected in crude extracts of *H. pylori*. Similar nucleases are identified in the membranes of Gram positive EndA and NucA are proteins that are required for Streptococcus pneumoniae (End A) and *Bacillus subtilis* (Nuc A). Another nonspecific endonuclease from *Anabaena sp*. (Nuc A) is capable of degrading single- and double-stranded DNA and RNA in the presence of divalent metal ions (Ghosh et al., 2005). Both DNA and RNA are substrates for EndA nuclease. EndA specifically degrades the non-transported strand (O'Rourke et al., 2004).

1.2.3 DNA extraction

The requirements for DNA extraction in crude and pure forms for different analytical and research purposes representing tens of disciplines that encompass gene cloning, gene bank constructions, DNA amplification, DNA recovery from rare samples, DNA sequencing, DNA storage, gene therapy, gene function studies, and their applications in forensic medicine and diagnostic microbiology. In most cases, simple and direct DNA extraction methods may be adequate for intended testing. However, some organisms including the helical bacteria *Helicobacter pylori*, *Campylobacter jejuni*, the isolate QUBC 70, and others, may present a serious problem since they appear to have been under estimated. Scant number of reports discusses DNA instability and the difficulties associated with manipulating DNA from helical bacteria. False negative results go unnoticed when searching for helical bacteria such as *H. pylori* in water, environmental, and clinical samples (Atteyeh, 2007), the absence of reports on false negative results is due to the difficulty of documenting negative results which may result from the poor quality of DNA used in DNA analyses; part of such false results is most likely due to nuclease activities. . Most workers have proceeded with their research utilizing available DNA extraction methods (Amundsen et al., 2009) or utilizing commercially supplied kits for DNA extraction. Unfortunately, only few researchers have acknowledged having a problem with DNA extraction from *H. pylori* and *C. jejuni* (Amundsen et al., 2009).

Helicobacter pylori DNA extraction from stool was reported by Makristathis and his colleagues in 1998; the procedure is far from simple since it entails several steps; it involved direct cell lysis, centrifugation, enzymatic digestion, boiling, and DNA purification by column chromatography (Makristathis et al., 1998), extracellular nucleases may create a problem as well (Chang et al., 1992). Nogva et al. (2000) used magnetic beads (Dynabeads, Oslo, Norway) for DNA isolation from *C. jejuni* resuspended in TE buffer (10 mM Tris, 1 mM EDTA), pH 8.0.

1.4 Detection of Nuclease activity

There are different methods for the detection of nuclease activity:

1.4.1 Agar diffusion

Deoxyribonuclease agar test strains were inoculated in agar plates supplemented with 2 g/l of herring sperm DNA. After 3 days of incubation, precipitation of nucleic acids was performed by adding 1N HCl to the plates for 5 minutes. Formation of hyaline halos around the colonies indicated DNase activity, in contrast to an opaque background for unhydrolysed DNA. Nuclease S1 from Aspergillus oryzae and an endonuclease from *Escherichia coli* (Sigma Chemical Co. MI, USA) were spotted on the plates prior to addition of HCl as controls for the DNA degradation and precipitation. The test is useful for differentiating *Serratia* from *Enterobacter, Staphylococcus aureus* from coagulase-negative staphylococci, and *Moraxella catarrhalis* from *Neisseria species*. This DNAase test agar depends on the method developed by Jeffries et al, 1957 (Jeffries et al., 1957; Weckman and Catlin, 1957) that a semi-synthetic medium with nucleic acid solution incorporated in the medium. Enzymatic activity is detected by flooding the plate with hydrochloric acid (1N HCl); a clear zone surrounding growth indicated a positive reaction. The DNase reaction helps in the differentiation and identification of non-pigmented *Serratia marcescens* (positive DNase reaction) from *Klebsiella-Enterobacter* (negative DNase reaction). Normal HCl is bactericidal and the organisms cannot be recovered from the surface of the agar after flooding. The incorporation of dyes into the medium which can distinguish hydrolysis of DNA is a useful modification which avoids the use of acid. Toluidine blue and methyl green form colored-complexes with polymerized DNA; such colors change as the DNA is hydrolyzed. It should be noted that toluidine blue inhibit Gram positive bacteria and is useful with Gram negative nuclease detection (as with *Enterobacteriaceae*). It has been used with ampicillin (30 mg/liter) to demonstrate DNase production by *Aeromonas hydrophila* from feces. Another modification was introduced; the metachromatic dye toluidine blue (Waller et al., 1985) used techniques in which the metachromatic dye toluidine blue O (TBO) concentration was reduced from 0.1% to 0.05% resulting less staining intensity, less masking of DNase-positive reactions due to over staining, and better contrast with sharper delineation of DNase activity zone, this gave a higher sensitivity of DNase detection.

1.4.2 Agar-dye diffusion

This modification of the Agar diffusion method (1.4.2 above) reduces dye content in the agar also enhanced expression of DNase activity by some organisms and provided sharper delineation of DNase-positive reactions. Rapid agar diffusion method used for the detection of DNase production by used brucella agar, a strong pink zone indicating DNA hydrolysis was seen around the wells after 20 to 24h of aerobic incubation at 37°C. Pretreatment of cells with polymyxin B, which released the cell-associated DNase, shortened the time needed to read positive results to 8 h and increased the zone size. This method as was used to detect DNase activity in *campylobacter* (Hanninen, 1989).

1.4.3 Fluorescent DNA

Fluorescent DNA was used for fluorometric detection of DNase activity analog (Takahashi and Ling, 1991). The enhancement of fluorescence of the DNA analog following nucleolytic degradation to mononucleotides was found to be a convenient signal for studying nucleases, especially exonucleases. This measurement, which is simple and sensitive, detects various kinds of DNase and can be applied to the detection of nucleases in the course of protein purification. The signal change can be observed continuously during the reaction and easily converted to the amount of liberated mononucleotide. The method is thus suitable for quantitative and kinetic studies of exonuclease activity.

1.4.4 Chromatin Digestion

DNase I activity can be monitored by following changes in chromatin structure that has been subjected to DNase digestion (Prentice and Gurley, 1983) .

1.5 Problem statement

The high activity of nucleases in helical bacteria hampers DNA isolation and utilization in all molecular based studies; cloning, diagnosis, sequencing, and storage of extracted DNA.

1.6 Justification

It is imperative that isolation of DNA from any source sample be 100% reliable and dependable in any molecular based method. Since the failure to obtain intact DNA suitable from targeted sources will inevitably lead to failed methods, results, and conclusion. It is strongly believed that this was the case in many reports regarding the presence and distribution of *H. pylori* in the environment and represents a current challenge to microbiologists (Atteyeh, 2007; Biesbroek et al., 2012; Makristathis et al., 1998).

1.7 Objectives

This research is important for many reasons:

1. Knowledge of the nucleases allows researchers and users of cell products to avoid the negative and undesirable effects of nucleases.
2. Searching for the biological function of an enzyme may lead to discoveries of biochemical pathways and the interaction of cell components to achieving cell function.
3. Identification of nucleases may reveal their role in virulence and pathogenesis of bacteria, it may help explaining unique characteristics of helical bacteria; difficulty in culture, subculture, storage, and morphological and genetic transformation.
4. Nucleases as unique enzymes may be targeted by antimicrobial agents for therapeutic purposes
5. Basic knowledge is enhanced and can be extended to other microbes that may share these genes.

1.8 Questions

2. What are the most efficient, fast and inexpensive method to extract bacterial DNA in the presence of these enzymes?
3. How does this enzyme degrade nucleic acids and where is it located in the cell, and what is its normal concentration?
4. How is the gene regulated and expressed?
5. How can the enzyme activity be controlled, enhanced, or inhibited?
6. Is there a standardized method for the isolation of these enzymes by cloning?

Chapter II

Methods

2.1 Isolation of Bacteria

Campylobacter jejuni ATCC 29428 (HY-Laboratories Ltd.), *Helicobacter pylori* ATCC 43526 (*H. pylori* ATCC 43526) and helical bacterium QUBC 70 were used in this study. The isolated bacterium QUBC 70 was similar to *H. pylori ATCC 43526* (Barghouthi, 2011), it was isolated from a stool sample of a patient suspected for having *H. pylori* gastritis. QUBC 70 grew under the same conditions and behaved as *H. pylori* and *C. jejuni*. However, unlike *H. pylori or C. jejuni*, QUBC 70 was able to grow at 35 ^{o}C, aerobically, and on nutrient agar (without blood). Efforts failed to obtain *H. pylori* from stool samples that were positive for *H. pylori* antigen collected from Sheik Zayed Hospital (Ramallah), and the Arab Health Center (Abu Deis), as. In addition attempts were made to isolate *H. pylori* from gastroscopy biopsies positive for *H. Pylori*, although few attempts were successful; the bacterium was fragile and was lost. Twenty stool samples, and two biopsies samples from gastroscopy were collected and grown on Skirrow's selective sheep blood agar (Skirrow's antibiotics; 10 µg/ml vancomycin, 5 µg/ml trimethoprim, 2.5 IU/ml polymyxin B, 100 µg/ml cycloheximide) according to Bargouthi (2009) or modified Skirrow's which is selective for *C. jejuni* known as Line (Eric et. 2000) used for culturing *C. jejuni, H. pylori*, and QUBC 70; it is a selective medium suitable for all three bacteria; Sheep

blood agar or chocolate agar made with blood agar base or brucella agar supplemented with Skirrow's antibiotics were used to culture all three bacteria (*C. jejuni*, *H. Pylori*, *or* QUBC 70); nutrient agar was used to culture QUCP70, *Staphylococcus aurues*, and all other bacteria used in this study (Dharmalingam et al., 2003); as reported by Bargouthi (2009).

2.2. Bacterial growth and cultures

Defibrinated sheep blood was collected in sterile large-mouth bottles containing glass beads from local slaughter house (Al-Bireh, Palestine) with shaking. Thirty ml-aliquots were treated with 2.5 ml of stock (200 x Skirrow's (Skirrow's: 10 µg/ml vancomycin, 5 µg/ml trimethoprim, 2.5 IU/ml polymyxin B, 100 µg/ml cycloheximide) or modified Skirrow's (10 µg/ml vancomycin, 5 µg/ml trimethoprim, 2.5 IU /ml polymyxin B, 100 µg/ml cycloheximide, and 10 µg/ml rifampicin (Line) previously made in 70% ethanol and stored at 4˚C). The treated aliquots were kept for 24h at 4˚C until used in preparation of blood agar using Blood Agar Base. Agar plates were used within 10 days; inoculated with helical bacterium and incubated at 36˚C for *C. jejuni* or *H. pylori* and 30˚C for QUBC70. As described above, aerobic growth on NA was usually applied to QUBC70. Incubation was carried out for 3 days.

2.3 Lysate preparation

Agar plates were used to collect bacterial lawns using 10 µl sterile plastic loops, bacteria was collected in autoclaved snap-top microfuge tubes containing 1.5 ml of autoclaved J-buffer (100 mM Tris–HCl, 100 mM EDTA, 150 mM NaCl; pH 8) (Barghouthi, 2011) or DNase buffer (0.1 mmol/L NaCl, 20 mmol/L $MgCl_2$ and 2 mmol/L $CaCl_2$; pH: 7.6) as stated for each method of lysis. Absorption of each bacterial suspension was determined at 600 nm (Spectronic 200) as following; to 990 µl of DNase or J-buffer, 10 µl of bacterial suspension was added and mixed by gentle pipetting (usually OD range was 0.06 to 0.4; low for *C. jejuni* and high for QUBC70) this was useful when equivalent aliquots were needed. The aliquots were then subjected to the selected method of lysis and experiments. Endogenous DNA was degraded by allowing lysate to incubate for 48 h at 36˚C.

2.3.1 Sonication

Bacterial cells were harvested in 1ml of DNase buffer and kept on ice (to slow the activity of enzyme), then sonicated 3 to 5 times for total DNase activity according to (Suslick, 1990). For extracellular DNase activity, bacteria were harvested in 1ml DNase buffer; centrifuged, washed with DNase buffer, then the supernatant was tested for extracellular DNase activity. For intracellular DNase activity, washed cells were sonicated using a microson (Ultrasound Cell disruptor probe, Misonix Inc, Microson XL Model DU-2000).

2.3.2 Lysozyme lysis

Bacteria collected in J-buffer as above (section 2.3) was treated with egg lysozyme 100 µl/ml (5mg/ml made in J-buffer and kept at 4˚C). Mixed and incubated at 36˚C for 10 min (Gram negative) or 30 min for Gram positive. Bacteria were then collected by centrifugation (at 13000 rpm for 2min; Hettich Zentrifugen, Mikro 12-24). The bacterial pellet was lysed in 100µl of 1% SDS diluted in sterile water, then incubated for 2-3 days at 36˚C.

2.4 Detection of DNAase activity

2.4.1 Agar diffusion assay

Acridine orange (Sigma Chemical Co.) was used to detect the activity of bacterial nuclease according to (Leith, 1963). Acridine orange agar plates were made with nutrient agar containing (1 mg/ml acridine orange) and 10 mg/ml salmon sperm DNA. The DNA was added before cooling or after cooling to generate ssDNA plates or dsDNA plates. The plates were then viewed under UV light for clear (dark) areas around the colonies.

2.4.2 Spectroscopy (Nanodrop)

Fresh lysates of *E.coli*, *S.aures*, QUCP70, or *Campylobacter* were used. DNase buffer was used to dilute Lambda DNA (Promega, 5µg/µl to achieve 0.1µg/ml). One µl aliquot from a mixture of 6 µl of each different lysate, 3µl of λ-DNA (~1.5µg) and 1µl of 10X DNase buffer (a total volume of 10 µl) was used for each time point reading (0.0, 30, 90, or 150 min) using the Nanodrop (1000 spectrophotometer, Thermo Scientific, Bancrot, USA).

The instrument produces OD ratio of 260/280 nm, as well as concentrations. Increased readings indicate degradation of DNA.

2.4.3. Degradation of DNA and agarose gel electrophoresis

Electrophoresis of DNA in 1% agarose made in TAE buffer (Tris Acetate EDTA, pH 8, prepared at 50X and autoclaved, then diluted to 1X with deionized (RO) water before use) allows the detection and profiling of DNA degradation. Intact λ-DNA, Hind III digested λ-DNA, exogenous bacterial DNA, or endogenous bacterial DNA was used as an indicator of DNase activity as detailed for each experiment below.

2.4.3.1 Endogenous and Exogenous bacterial DNA digestion

Lysate were subjected to different incubation conditions to show that digestion of endogenous DNA (showing high viscosity lysate) lose their viscosity when endogenous DNA is degraded. Degradation was checked by agarose gel electrophoresis. In other experiments, a small fraction of the lysate (3-5 µl) was added to exogenous bacterial DNA extracted from *Escherichia coli* as discussed in details by (Barghouthi et al., 1991), briefly lysozyme treated bacteria was lysed in SDS and treated with pronase, dialyzed against 10mM EDTA, then phenol extracted, isoamyl-chloroform extracted, dialysed again and assayed at 260/280nm for purity. The qualitative assay compared the effect of different lysate under different conditions on the added *E. coli* DNA using agarose gel electrophoresis.

2.4.3.2 Lambda DNA intact or Hind III digested

The deoxyrebiunclease activity of different lysate preparations was also assessed using intact lambda (λ) DNA; 50 kbp intact lambda DNA was tested using nanodrop method. However a better strategy was to visualize Hind III digested λ- DNA banding pattern before and after DNAase treatment. The change in pattern then can be evaluated for it may reveal the type of nuclease being sought.

2.5 Kinetics of DNA degradation

This experiment was designed to check for the time required for digestion by the lysate. Equivalent aliquots of a single lysate (clear supernatant preparation) were kept frozen at -

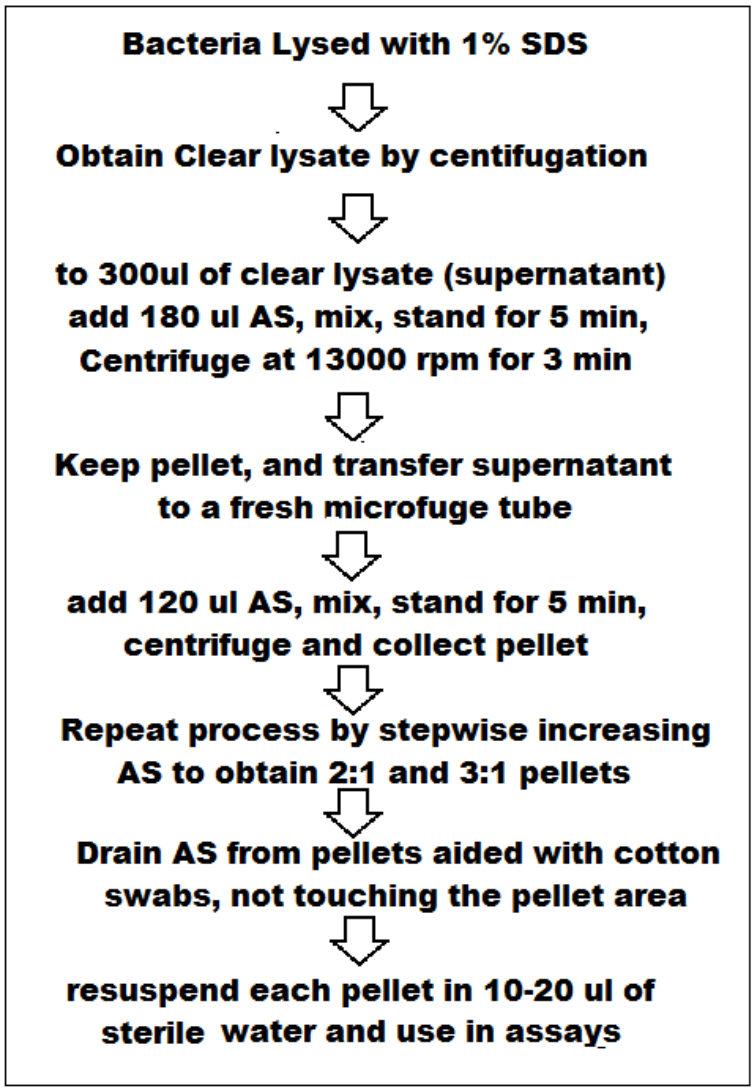
20˚C. First tube representing the longest incubation time was prepared first and incubated at 36˚C (48h), after 24h, a second tube was incubated to allow a 24-h incubation, after 44h a third tube was prepared to allow for 4-h of incubation, after 46h a fourth tube (2-h incubation), after 47.5 h a fifth tube was incubated allowing a 30 min incubation. After all tubes were incubated for the different lengths of time (i.e. 30 min, 2 h, 4 h, 24 h, and 48h) each tube received 100µl Na-acetate pH 4.8 and 100µl isopropanol for 30min at room temperature, centrifuged, and pellets were washed with 70% ethanol, dried at ambient temperature, then resuspended in 10µl of pure sterile water. Four µl of each were loaded per well of agarose and visualized under UV light. Variations of the method were used as indicated in the results section.

2.6 Inhibition method

Attempts to add potential inhibitors to lysate included SDS, 2-mercaptoethanol, RNase, Phenol, Proteanase K, or pronase were all tested.

2.7 Cell fractionation using ammonium sulfate method

A saturated water solution of ammonium sulfate (AS) was prepared at 25˚C and allowed to equilibrate in presence of excess AS. The saturated AS solution was mixed with supernatant of cleared bacterial lysate; AS: lysate at 1: 1 (V/V) is designated as 100% mixture, 2 AS: 1 lysate (V/V) is designated 200%, 3:1 (V/V) is designated 300%. On the other hand 1:2 V/V is designated 50%, and 0.6:1 (60%) to achieve different percentages. The procedure was carried out by addition of small amount of AS to the lysate (e.g. 25%) mixed well, allowed to stand for 5 min then centrifuged for 3 min at 13000 rpm. The pellet after AS precipitation was kept after tubes being completely and carefully drained. To the supernatant that was transferred to a fresh tube additional AS was added gradually to a higher percentage. The process was successively repeated till 300% was achieved. Each fraction was then tested for DNase activity and analyzed by SDS_polyacrylamide gel electrophoresis (SDS-PAGE). The steps are summarized in the following flow chart:



Campylobacter jejuni was fractionated into 3 fractions as following: Cells were washed and collected in 1 ml J-buffer ($600_{nm\ 1\%}$= 0.075), lysozyme treated, lysed in 500 µl sterile water, and then separated into soluble fraction (A1; cytosol fraction and viscous/pellet B1; cell envelope and chromosome). B1 was dissolved in 50 µl of 1% SDS and diluted with 450 µl sterile water. A1 and B1 were centrifuged for 5 min at 13,000 rpm. Pellet from A1 was discarded; pellet from B1 was suspended in 500 µl water and labeled as (C1). The 3 tubes were sealed with parafilm and incubated at 36˚C for 45 h. Viscosity in tube B1 was lost after this incubation. 100% AS precipitation was carried out with fractions A1 and B1. The AS pellets of A1 or B1 were resuspended in 50 µl of HindIII buffer-E (Promega), 3 µl of A1, B1, or C1 were used in digestion experiments.

2.8 Protein profiling by SDS-PAGE

Sodium dodcyl sulphate Polyacrylamide gel electrophoresis was prepared as 10% according to (Harlow and Lane, 1988), for 20 ml gel (7.9 ml H_2O**,** 6.7 ml 30% acrylamide mix, 5 ml Tris-1.5M, pH8.8, 0.2ml SDS (10%), 0.2ml ammonium persulfate (10%), and 1µl TEMED), after casting and polymerization, stacking acrylamide (5%) was casted; for 8 ml of stacking gel (mix: 5.5 ml H_2O, 1.275 ml of 30% acrylamide mix,1.25ml Tris -1M-pH6.8, 1ml SDS (10%), 1ml ammonium persulfate, TEMED -1µl). Test sample (2:1) was mixed with loading buffer (50mM Tris pH 6.8, 2% SDS, 1% bromophenol blue, 10% glycerol) and was loaded onto the SDS-PAGE gel. Electrophoresis was performed. Fixation and staining with Coomasi blue prepared in ethanol and acetic acid for 15 min followed by destining in destaining buffer (30% methanol, 10% acetic acid).

2.9 Protein determination using Bradford reagent

Protein was determined using Bradford method (Bradford, 1976).To 33 µl of protein sample or lysate, 967 µl of Bradford reagent was added; a standard curve of bovine serum albumin was prepared and run simultaneously together with a blank (976 µl Bradford to 33 µl of DNase buffer). After 30 min of incubation at 37 ^{o}C, absorption was determined at 580nm (the protein dye complex causes a shift in the absorption maximum of the dye from 465 to 595nm) (spectronic 200).

Chapter III

Results

3.1 Growth and identification of QUBC70

The bacterium QUBC 70 has been discovered by chance, during attempts to isolate *H. pylori* from *H. pylori* antigen-positive stool sample. QUBC 70 has a helical shape similar to *Helicobacter* (Figure 3.1).

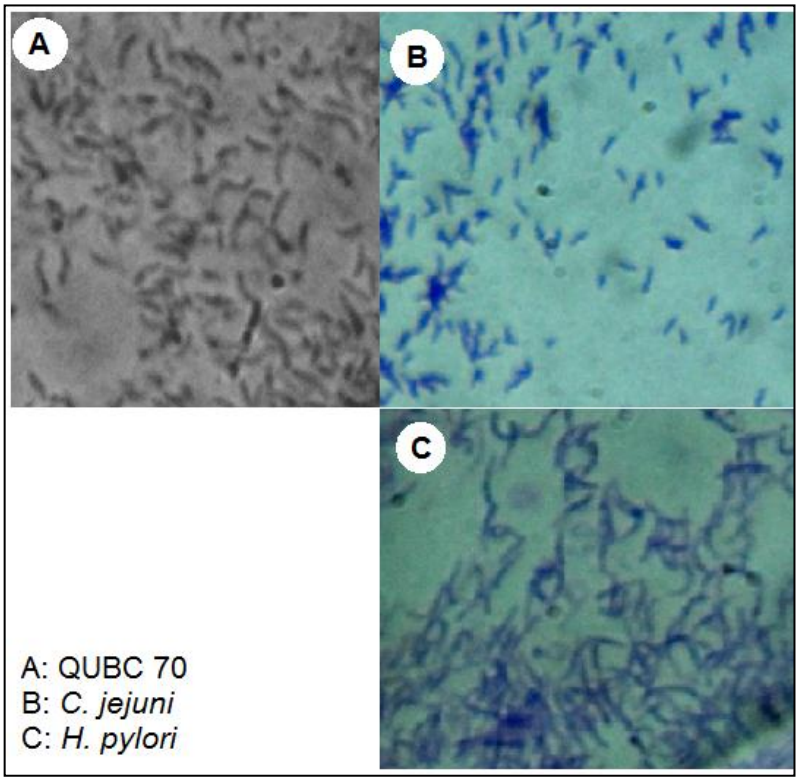
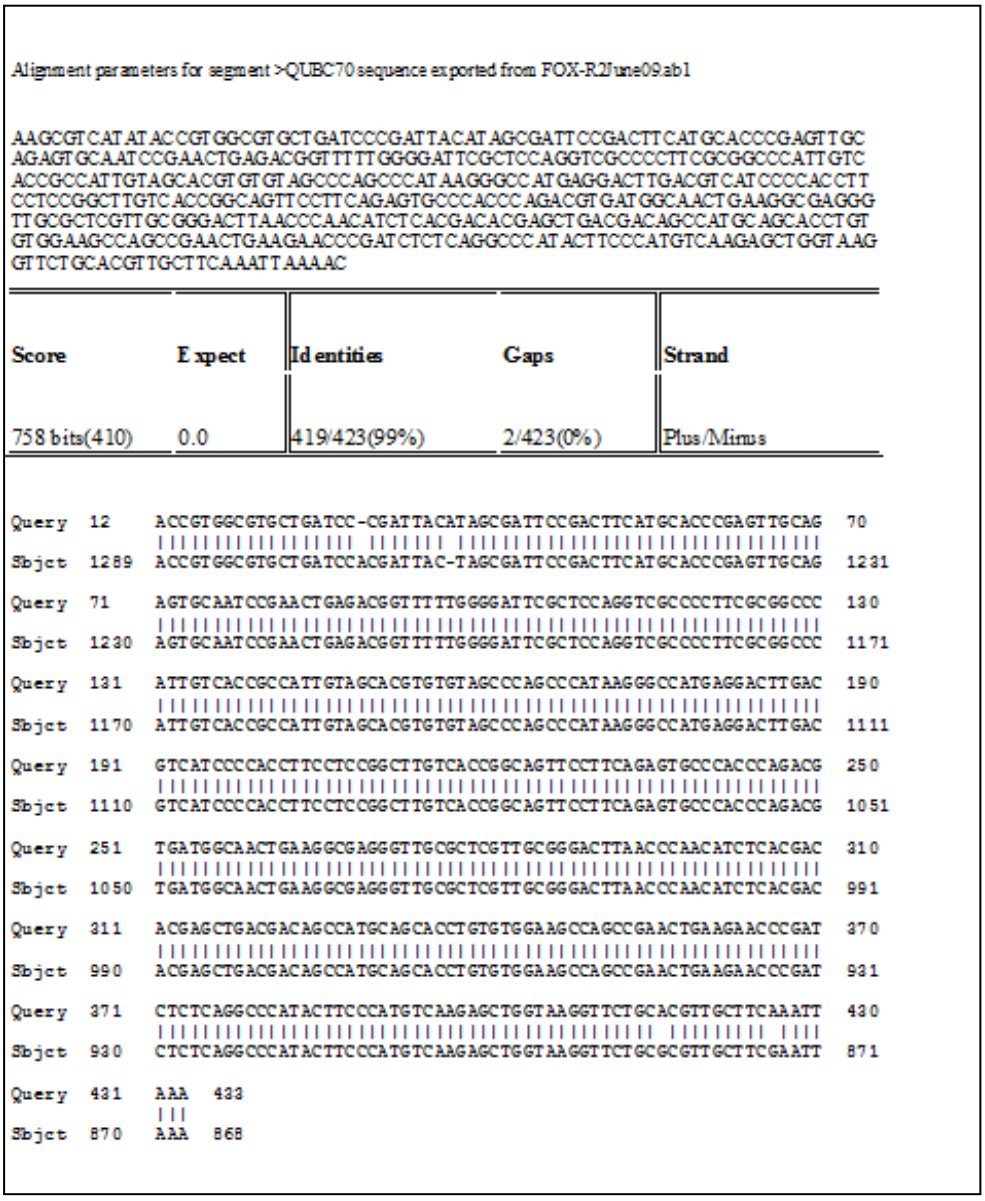


Figure 3.1: Staining of heat fixed fresh colonies of bacteria with crystal violet. Air dried smears of QUBC70 (A), C. *jejuni* (B)*;* and *H. Pylori* (C). Helical bacteria appear as fusiform with tapered ends, S-shape, or curved rods, photographed directly from light microscope (1000X).

Attempts to identify QUBC 70 using *H. pylori* specific PCR primers failed to identify the bacterium. The bacterium can be grown on nutrient agar (NA) supplemented with Skirrow's antibiotics (Bargouthi, 2009), or modified Skirrow's (Line). It was found to be facultative microaerophilic that produced good growth on NA with or without selection within 48 hours, it remained viable on agar plates for >1 week when kept at 4 ^{o}C. The universal method identified the bacterium as was carried out for this bacterium, the DNA sequence of the 16S PCR amplicon indicated QUBC70 to be closely related to an Alpha proteobacterium strain PIII -38b or strain H1 (99% BLAST shown below; (Barghouthi, 2011).



Alignment parameters for segment >QUBC70 sequence exported from FOX-R2June09.ab1

AAGCGTCATATACCGTGGCGTGCTGATCCCGATTACATAGCGATTCCGACTTCATGCACCCGAGTTGC
AGAGTGCAATCCGAACTGAGACGGTTTTTGGGGATTCGCTCCAGGTCGCCCCTTCGCGGCCCATTGTC
ACCGCCATTGTAGCACGTGTGTAGCCCAGCCCATAAGGGCCATGAGGACTTGACGTCATCCCCACCTT
CCTCCGGCTTGTCACCGGCAGTTCCTTCAGAGTGCCCACCCAGACGTGATGGCAACTGAAGGCGAGGG
TTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCAGCACCTGT
GTGGAAGCCAGCCGAACTGAAGAACCCGATCTCTCAGGCCCATACTTCCCATGTCAAGAGCTGGTAAG
GTTCTGCACGTTGCTTCAAATTAAAAC

Score	Expect	Identities	Gaps	Strand
758 bits(410)	0.0	419/423(99%)	2/423(0%)	Plus/Minus

```
Query  12    ACCGTGGCGTGCTGATCC-CGATTACATAGCGATTCCGACTTCATGCACCCGAGTTGCAG  70
             |||||||||||||||||| ||||||| ||||||||||||||||||||||||||||||||||
Sbjct  1289  ACCGTGGCGTGCTGATCCACGATTAC-TAGCGATTCCGACTTCATGCACCCGAGTTGCAG  1231

Query  71    AGTGCAATCCGAACTGAGACGGTTTTTGGGGATTCGCTCCAGGTCGCCCCTTCGCGGCCC  130
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1230  AGTGCAATCCGAACTGAGACGGTTTTTGGGGATTCGCTCCAGGTCGCCCCTTCGCGGCCC  1171

Query  131   ATTGTCACCGCCATTGTAGCACGTGTGTAGCCCAGCCCATAAGGGCCATGAGGACTTGAC  190
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Sbjct  1170  ATTGTCACCGCCATTGTAGCACGTGTGTAGCCCAGCCCATAAGGGCCATGAGGACTTGAC  1111

Query  191   GTCATCCCCACCTTCCTCCGGCTTGTCACCGGCAGTTCCTTCAGAGTGCCCACCCAGACG  250
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Sbjct  1110  GTCATCCCCACCTTCCTCCGGCTTGTCACCGGCAGTTCCTTCAGAGTGCCCACCCAGACG  1051

Query  251   TGATGGCAACTGAAGGCGAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGAC  310
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Query  311   ACGAGCTGACGACAGCCATGCAGCACCTGTGTGGAAGCCAGCCGAACTGAAGAACCCGAT  370
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Sbjct  990   ACGAGCTGACGACAGCCATGCAGCACCTGTGTGGAAGCCAGCCGAACTGAAGAACCCGAT  931

Query  371   CTCTCAGGCCCATACTTCCCATGTCAAGAGCTGGTAAGGTTCTGCACGTTGCTTCAAATT  430
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Sbjct  930   CTCTCAGGCCCATACTTCCCATGTCAAGAGCTGGTAAGGTTCTGCGCGTTGCTTCGAATT  871

Query  431   AAA  433
             |||
Sbjct  870   AAA  868
```

Figure 3.2 BLAST result. Alpha proteobacterium H1(2012) 16S ribosomal RNA gene, partial sequence Sequence ID: gb|JN693496.1|Length: 1408Number of Matches: 1, Related Information, Range 1: 868 to 1289GenBankGraphics

3.2 Detection of Nuclease activity

3.2.1 Diffusion assay in acridine Orange

The agar diffusion method was tested and soon abandoned due to its insensitivity, requirement for large amounts of DNA (>30mg DNA per 15 ml-plate), it is slow, not applicable for bacteria that require blood agar or chocolate agar because clear area could not be seen.

3.2.2 Degradation of endogenous DNA in bacterial lysate

As bacterial cells were lysed, the Nucleases and other enzymes started to digest any possible substrates unless inhibited by other physical or chemical factors (Nucleases are usually inhibited by SDS or degraded by proteases or the addition of metal chelating agents such as EDTA). Figure 3.3 shows that endogenous DNA of QUBC70 was degraded slowly (the lambda DNA is apparent as a band just below the well in the control and 1 h lanes). A long streak of degraded DNA was observed after 3h of incubation. Longer periods of incubation (24 and 48 h) resulted in near complete degradation. Such enzyme behavior indicated low concentration/ rate of degradation. Yet, it indicated that the putative nuclease is highly stable and may resist proteolyses degradation by endogenous protease. Pronase treatment of QUBC70 lysate during the long procedure for DNA preparation had failed to protect the DNA from being degraded and the DNA was lost during dialysis (Barghouthi et al., 1991).

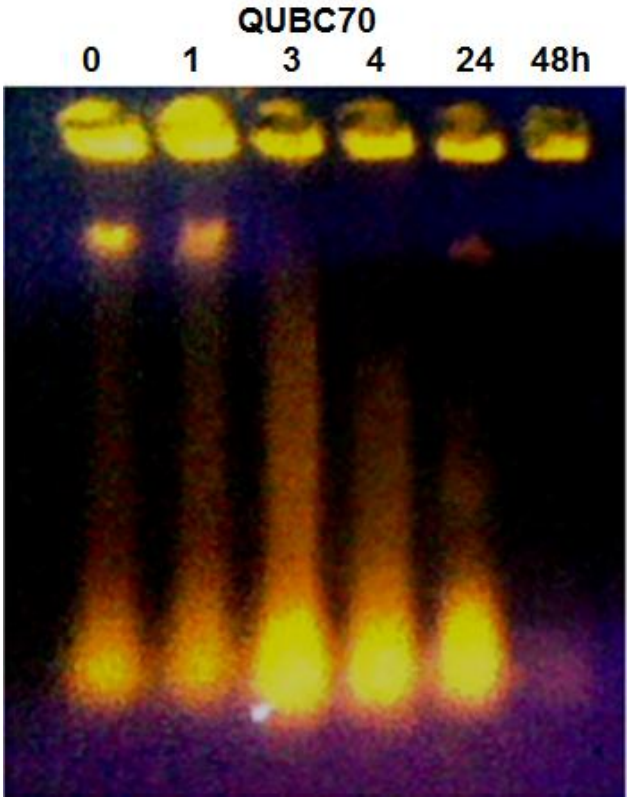

Figure 3.3: **Agarose gel electrophoresis of QUBC70 lysate with time**. QUBC70 lysate was incubated at 36 ^{o}C for different periods of time (h) immediately after lysis. Note the streaking of homologous DNA after 3h and the complete degradation after 48h. DNA was not degraded during the first 30 min (lanes 0 and 1) showing the DNA remaining in or just out of the wells.

3.2.3 Degradation of λ-DNA by different bacterial extracts

Analysis of bacterial lysate against λ-DNA confirmed the original observation of the presence of DNase activity that was disruptive to obtaining stable DNA from helical bacteria. The results shown in Table 3.1 and presented in Figure 3.3 supported the presence of a nuclease that caused an increase in the 260/280 absorption ratio as determined by the Nanodrop spectrophotometer.

Table 3.1: Measurement of nuclease activity using Nanodrop method. Lambda DNA (Control), was added to the lysate prepared in DNase buffer and incubated for indicated times at 36C. Evidence of Nucleic acid and protein degradation was observed as the optical density of both 260nm and 280nm increases with time of incubation in sonicated QUBC70 and *Campylobacter*. ng/ul represents the concentration of DNA.

Time	Sample	A-260	A-280	ng/ul
zero	Control	1.181	1.007	98
30 min		3.089	3.637	337.4
1.30 hr		5.20	2.269	260.5
2.30 hr		4.217	2.288	211
Time zero	*E.coli (son)*	6.181	3.99	309.1
30 min		5.511	4.031	275.6
1.30 hr		7.399	5.269	370
2.30 hr		6.389	4.494	320
zero	*E.coli (lys)*	4.771	3.443	238.6
30 min		7.214	3.975	360.7
1.30 hr		5.724	4.242	286.2
2.30 hr		5.123	3.088	256.1
zero	*S.aures(son)*	6.653	4.963	332.7
30 min		7.102	4.823	355.1
1.30 hr		7.548	5.824	377.4
2.30 hr		6.612	4.113	331
zero	*S.aures(lys)*	10.401	8.114	520.1
30 min		8.451	4.99	422.5
1.30 hr		24.962	19.336	1248.1
2.30 hr		4.655	2.576	233
zero	*QUBC70 (son)*	12.183	7.466	609.1
30 min		27.487	19.929	1374.4
1.30 hr		26.271	19.048	1313.6
2.30 hr		30.02	21.796	1501
zero	*QUBC70(lys)*	8.371	5.632	418.5
30 min		4.655	2.576	233
1.30 hr		35.396	25.66	1769.8
2.30 hr		7.13	4.082	356.9
zero	*Campy(son)*	14.866	9.277	743.3
30 min		16.519	10.705	825.9
1.30 hr		16.872	10.518	843.6
2.30 hr		20.083	13.381	1004.1

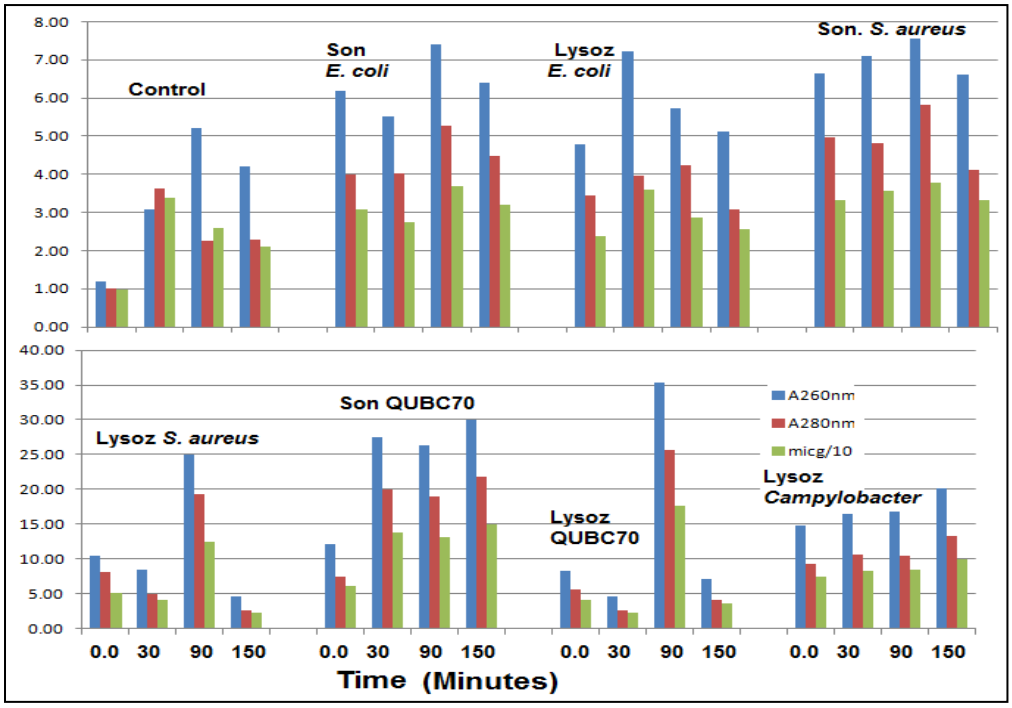

Figure 3.4: Absorbance of Lambda phage DNA incubated with bacterial lysate. Lambda phage DNA (1 µg in a final volume of 10µl DNase buffer (control), or a similar volume (10µl) containing the bacterial lysate (6µl). A sample was read after incubation at 36˚C for the indicated times (X-axis; Table 3.1). Lysoz (lysozyme), Son (sonicated).

3.3 Degradation of exogenous (*Escherichia coli*) DNA and endogenous DNA by QUBC70 SDS-lysate

Incubation of lysate of QUBC70 at 4˚C slowed the degradation of endogenous DNA. This has been shown repeatedly. Exogenous *E.coli* DNA was also degraded when using QUBC70 lysate as illustrated in Figure 3.5. The figure shows that the SDS lysate was active in degrading endogenous DNA (lane 10). Degradation was prevented at 4˚C (lane 12). Exogenous *E. coli* DNA which appeared as long streaks (dsDNA lane 8 or ssDNA) was not sensitive to degradation when treated with whole lysate (lane 11 relative to lane 8).

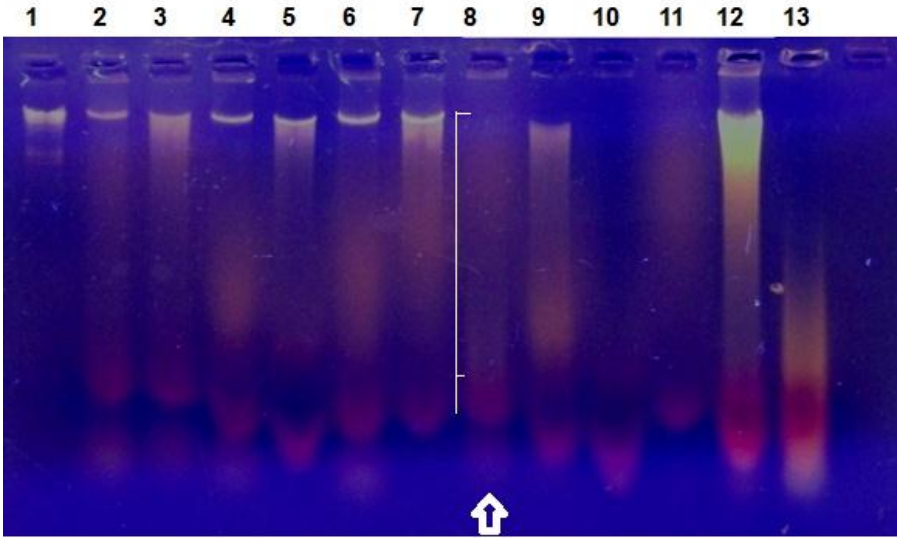

Figure 3.5: Degradation of exogenous DNA by lyates. *E.coli* control DNA was added as ds (lane 8) or ss (lane 9) to AS precipitated fraction (15%; lanes 2 ds& 3ss) or (35%AS Lanes 4ds &5ss) (50% AS lanes 6ds&7ss). Effective QUBC70 whole lysate endogenous degradation is seen in lane 10 or exogenous *E. coli* DNA degradation in lane 11. Control lysate was kept at 4˚C (lane 12) and endogenous DNA degradation is seen in lane 13. SDS lysate of QUBC70 were used in this experiment at 36˚C for 45h.

3.4 *Campylobacter* DNAase activity against 5'-staggered (5'-A↓A G C T T-3') end of λ -HindIII DNA or blunt end PCR amplicon

Extracted lysate of *C. jejuni* were tested for their ability to degrade blunt end dsDNA prepared by PCR amplification (Figure 3.6). *C. jejuni* nuclease activity extracted after lysozyme treatment and water lysis, viscous fraction was sheared with 23G needle till viscosity was reduced (vII; 10 µl of vII was kept for testing), the sheared lysate (~290 µl) was precipitated with 2-volumes (200%) of saturated AS after centrifugation the top supernatant layer (ASt) and the pellet were kept at 4 ^{o}C. Only 2 µl of extracts (vII and ASt) were used per digestion reaction of λ-HindIII DNA (0.33 µg/10µl reaction) or the 520 bp PCR amplicon (2.5µl of the amplified DNA). Two incubation temperatures were used (36 ˚C and 45 ˚C). The results indicated that a large fraction of the nuclease activity was removed from the ASt as evident in lane 2, Figure 3.6 showing incomplete digestion of λ-HindIII DNA relative to vII (36 ˚C; lane 3) which showed complete digestion of the λ-HindIII DNA. At 45 ˚C, ASt (lane 4) digested the λ-HindIII better than at 36˚C (lane 2). However, the two fractions poorly digested the blunt-end PCR amplicon at 36˚. Digestion with fraction vII treated amplicon at 45˚C was not complete as well, indicating that blunt end DNA may resist degradation relative to the more susceptible HindIII-DNA. The slow

movement of the smeared PCR amplicon in ASt-treated lanes (both temperatures) indicates a result similar to those obtained by Gel Retardation Assays.

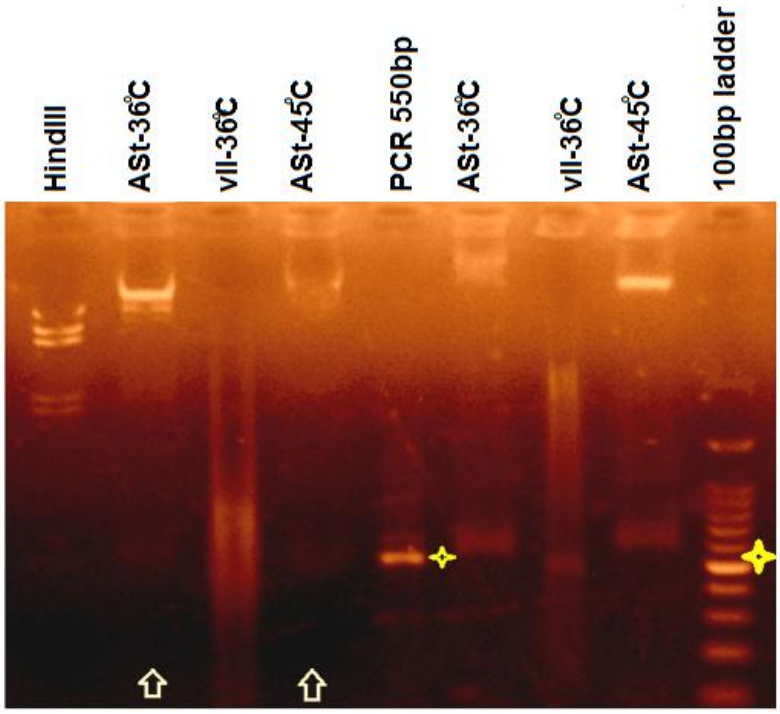

Figure 3.6: Digestion of blunt end vs. 5'-sticky end HindIII DNA. Degradation was more evident at 45 ˚C than at 36 ˚C. Nuclease activity was evident with blunt end (PCR amplicon), staggered end (λ-DNA) 5'-A↓A G C T T-3' [5´-sticky end] C. *jejuni* Nuclease activity was more evident at 45 ˚ than at 36 ˚C

3.5 Lysate dilution and nuclease activity

The concentration of the putative nuclease appeared to be extremely low, this observation was supported further by the dilution experiment (Figure 3.7) and lowering the incubation time to only 4h at 36˚C. As shown in Figure 3.7, only the undiluted extract was able to show some degradation of the HindIII-DNA with no streaking. The diluted sample was ineffective as seen after 4h of incubation.

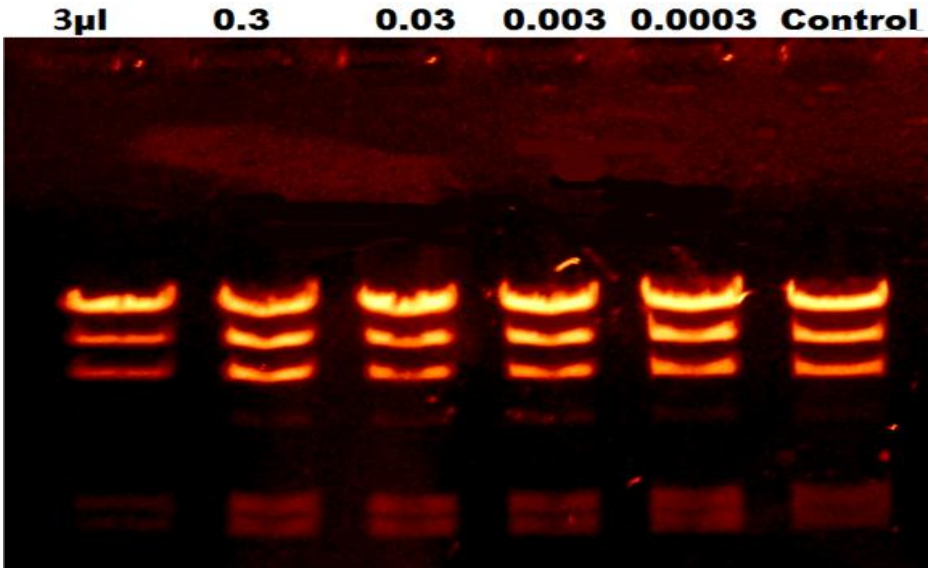

Figure 3.7:. Low concentration and slow action of the putative deoxynuclease at ten-fold serial dilution of *C. jejuni* cell lysate. The left lane is the only lane showing degradation within 4h. The remaining lanes were unaffected, indicating the action was enzymatic and not due to metal induced breakdown. The absence of smearing in the left lane strongly suggests an Exonuclease Activity.

Additional support was obtained when the experiment was repeated with AS 100% precipitated fraction of *C. jejuni* (to obtain a partially purified activity and to remove all soluble components that are not precipitated by 100% AS; 1: 1 v/v). The results in Figure 3.8 showed that 18-fold dilution from 1.5 to 0.083 µl of precipitated fraction (made in 10 µl of Hind III /E buffer) digested DNA within 45h, further 4-fold dilution to 0.0208 µl showed no activity; the diluted extract failed to digest target lambda DNA relative to the control (Crl; Figure 3.8), activity was lost with dilution indicating the absence of metal-induced degradation.

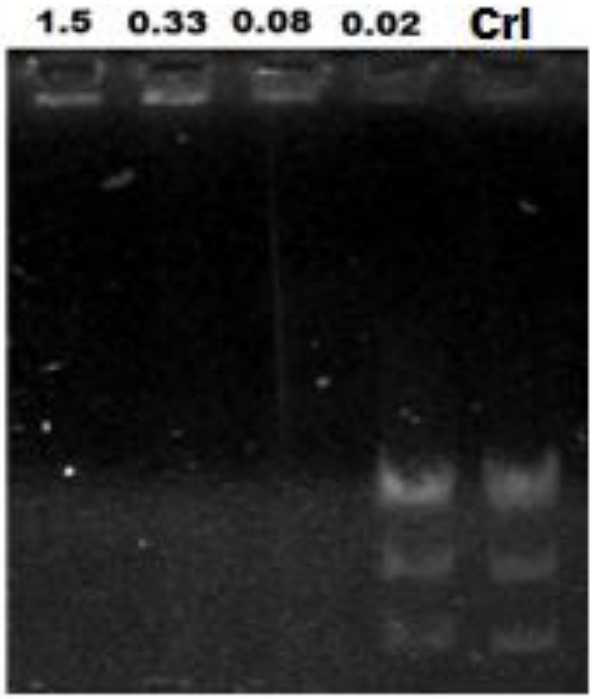

Figure 3.8: Ammonium sulfate Precipitation of C. jejuni lysate. Ammonium sulfate:*C. jejuni* lysate (1:1); (1.5µl) was serially diluted in Hind III(E) buffer (Promega Co.) containing λ-Hind III DNA (0.25 µg per 10 µl reaction volume) prepared and incubated at 36˚C in PCR thin-wall tubes, only 0.1 µg of DNA was loaded on the agarose). Lanes contained the indicated amount of AS fraction1.5 µl; 0.33 µl(dilution factor1/4.5); 0.083µl (1/4); 0.02 µl (1/4).

3.6 Fractionation of bacterial cells

Campylobacter jejuni was fractionated into 3 fractions A1 (cytosol), B1 (cell envelope and chromosome), and C1 (insoluble debris; see methods). The AS pellets of A1 or B1 were resuspended in 50 µl of HindIII buffer-E, 3 µl of A1, B1, or C1 were used in digestion experiment. The results (Figure 3.9) showed that A1 was not effective at 36˚C caused the λ-DNA to shift producing blurred bands yet A1 performed well at 45˚C. Fraction B1 was equally effective at 36˚C and 45˚C. However, fraction C1 (cell wall and cell debris insoluble part) had no clear evidence of nuclease nor of metal activities; it served as a good negative control. The importance of these results of this experiment is the suggestion of the presence of at least two separable nucleases, a soluble nuclease and an envelope or DNA associated nuclease.

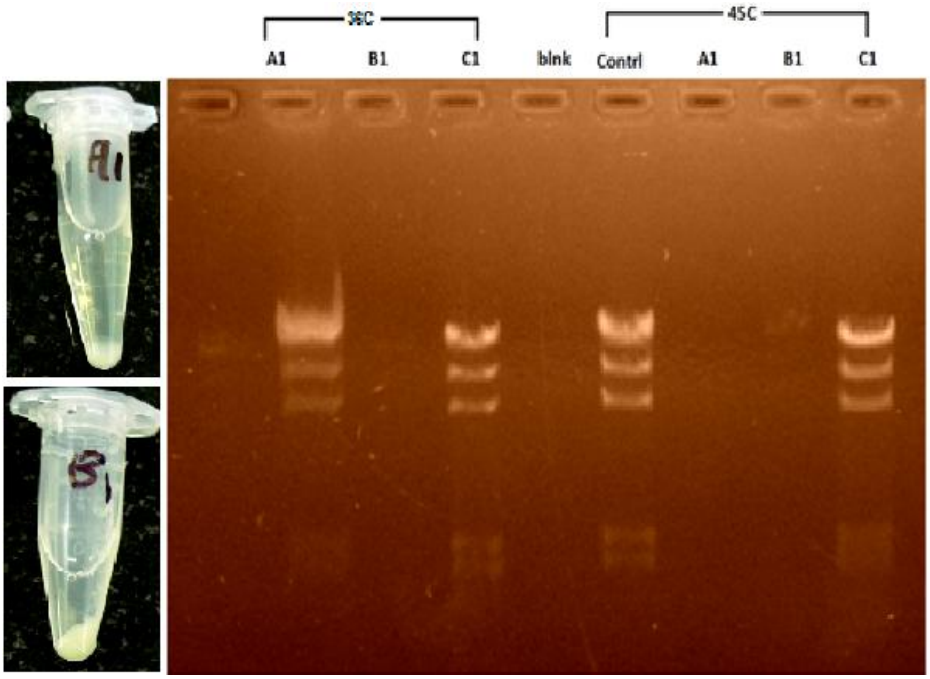

Figure 3.9: Agarose gel electrophoresis of digested λ-HindIII at 36˚C or 45˚C of *C. jejuni* AS fractions. *Campylobacter jejuni* cell envelope and soluble (cytosol) fraction microfuge tubes showing the AS precipitated components are shown. *Campylobacter jejuni* Cell envelope (B1) and soluble (cytosol) fraction (A1) were tested along with the SDS insoluble fraction (C1) that remained after B1 solubilization with SDS. The agarose gel; digestion of λ-HindIII at 36˚C or 45˚C with *C. jejuni* AS fraction A1, B1 or C1 after 45h of incubation in PCR tubes in the thermocycler with heated lid (MiniCycler, MJ Research, Inc., Watertown, MA).

3.7 Inhibition of Nuclease activity

Although not fully investigated, several attempts were made to inhibit DNA degrading activity associated with bacterial lysate had mostly failed indicating resilient putative nuclease(s). Failing list included 1% SDS, 2-mercaptoethanol, RNase, Phenol, 2-mercaptoethanol, Proteinase K, or pronase were all tested. RNase A was also negative at both temperatures (36˚C and 45˚C, not shown). No conclusive inhibitory effect was recorded. However, at 4˚C or 50˚C the activity was immensely reduced but not irreversibly inhibited (Figure 3.10 and 3.11).

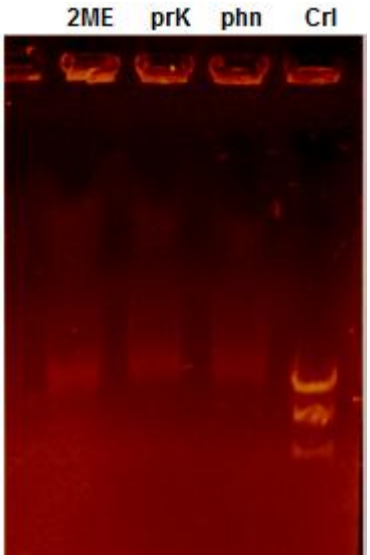

Figure 3.10: Effect of mercaptoethanol, protinase K and phenol on nuclease activity. Ammonium sulfate fraction of *C. jejuni* Cell envelope (B1) was utilized in this experiment after 45 h at 36˚C showing no inhibition of degradation relative to the control (Crl) in the presence of 2-mercaptoethanol (2ME), proteinase K (prK), or phenol (phn).

3.8 Fractionation of whole cell lysates

It was observed early in this work that lysozyme treated cells lysed with water and cleared by centrifugation, in the absence of SDS (cell envelope removed as cell debris) contained very weak nuclease activity, therefore, it was decided that SDS treatment was an important step in the process of preparing bacterial lysates that would release the nuclease activity.. When cells were fractionated into soluble and pellet fractions, significant activities were found in both cytosol and pellet fractions as evident in Figure 3.9. It was decided that whole cell lysate must be used before fractionation. This was accomplished by lysozyme-SDS treatment of cells, incubation at 36˚C for 45h to allow endogenous DNA degradation, centrifugation to obtain clear cell lysate suitable for AS precipitation.

3.9 Ammonium sulfate fractions and nuclease activity

Whole SDS cell lysate of *C. jejuni* or QUBC70 were incubated for 45h at 52˚C to allow degradation of endogenous DNA and cleared by centrifugation. AS precipitated as shown in Figure 3.9 (20, 40, 60, 80, 100, 200, and 300%) i.e. sequentially treated with increasing volumes of saturated AS. Each of the pellets was then resuspended in 20µl of HindIII

buffer and assayed for nuclease activity (3 µl per 20 µl reaction), the results are shown in Figure 3.11.

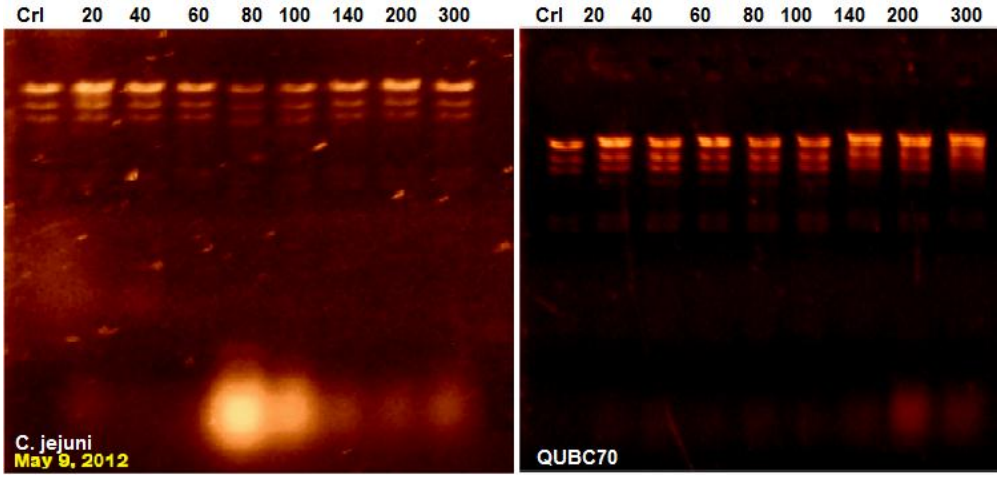

Figure 3.11: Effect of temperature on nuclease activity. Activity appeared to be reduced when lysate were kept for 45h at 51.7˚C and tested for an additional 45h at 50˚C. AS fractions performance was consistent with previous results. *C. jejuni* AS fractions (60-100%) especially 80% fraction showed degradation activity. QUBC70 activity was confined to AS fractions above 180% (i.e. 200 and 300%). Fractions collected with 400% did not show any precipitation.

To further confirm the previous AS fractionation. To 500 µl of clear lysate (SDS after Lysozyme) sequential precipitation was carried out. Precipitates were re-suspended in 50 µl of HindIII buffer, 1.5 µl of AS fraction/reaction were mixed with λ-HindIII DNA. Agarose analysis was carried out after incubation at 36˚C for 45h (Figure 3.11). AS fractions (25 and 50%) showed efficient degradation of λ-HindIII DNA, trace activity was still contained in the 100% showing a level almost similar to the original lysate, the positive control (+crl). QUBC70 extracted with 25% AS had no effect and is distinguishable from *C. jejuni* in this regard; the nuclease activity from QUBC70 repeatedly precipitated above 180% AS (Figure 3.12).

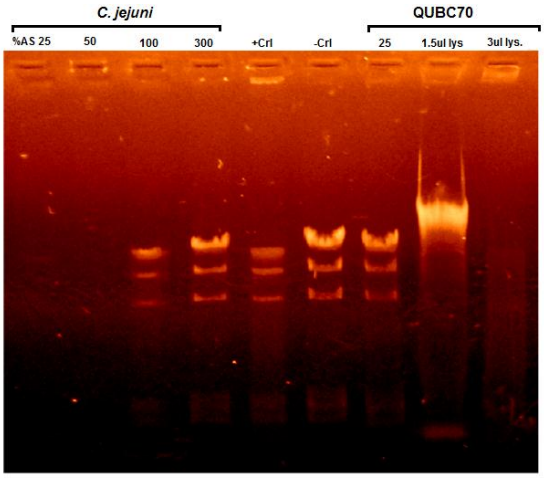

Figure 3.12: Ammonium Sulfate fractionated whole cell lysate. Fractions under 100% AS of C. jejuni showed substrate degradation. QUBC70; 25% AS was ineffective whereas fresh lysate 1.5 µl and 3 µl of an old preparation were effective.

3.10 Protein profiles of AS fractions on SDS-PAGE

Figure 3.13 shows all the proteins that can be AS precipitation from both *C. jejuni* and QUBC70; some proteins that present at concentrations below the PAGE detection limits may not be visible. Panel I shows the protein profile of 100% AS fractions obtained from the cytosolic fraction (A1) or the cell envelope fraction (B1) of both bacteria (*C. jejuni* and QUBC70), since AS fractions obtained below 100% were devoid from Nuclease activity, the nuclease from QUBC70 cannot be any of the shown proteins. This important result allows the exclusion of all bands obtained below 180% AS. The bands shared by fractions 200% or less can be excluded as well (Panel II). The asterisk in panel II indicate AS fractions showing nuclease activity; 60% and 80% for *C. jejuni*; 200% & 300% for QUBC70. The arrows pointing to protein bands of QUBC70 that may potentially contain the nuclease activity.

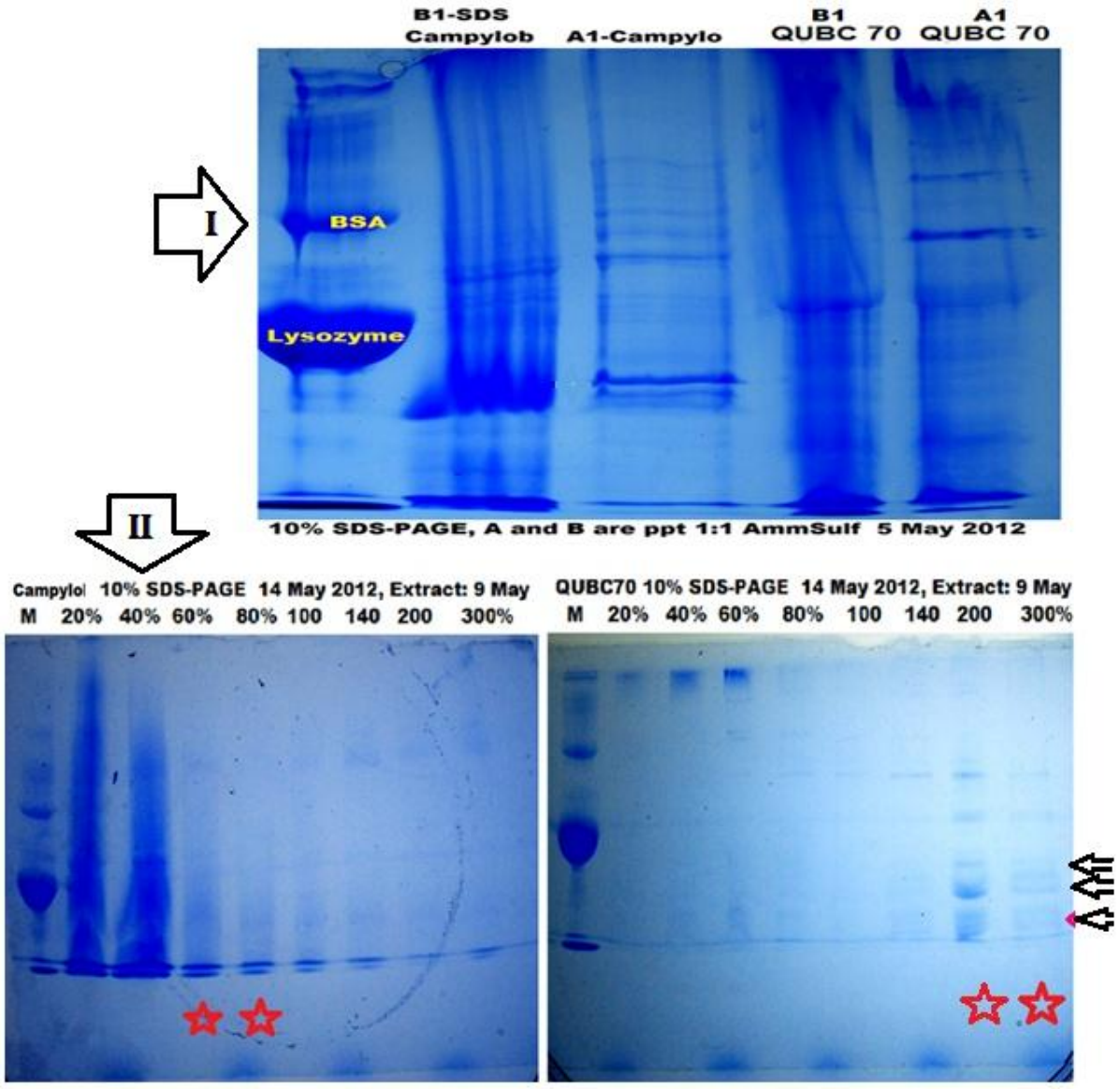

Figure 3.13: SDS-Polyacrylamide gel electrophoresis of bacterial proteins with nuclease activity. Panel I showing most proteins that can be precipitated with 100% AS from both bacteria cytosol fraction (A1) and the cell envelope fraction (B1). **Panel II** the stars mark the AS fractions that showed nuclease activity, all AS fractions are represented. Molecular weight markers; BSA, 66kDa and egg lysozyme, 14.3kDa.

Chapter IV

Discussion

4.1 Enzyme or metal

The results presented in Figure 3.6 with serial dilution of *C. jejuni* lysate, suggested that a nuclease rather than a metal was responsible for the degradation since a metal would have been present in all reactions. The results also suggest that the enzyme is most likely an exonuclease rather than an endonuclease due to the lack of smearing and the clean disappearance of the bands (i.e. one molecule at a time is being degraded from one end to the other end as indicated by the lighter bands observed with the higher putative enzyme concentration.

Additional evidence; none of the experiments carried out, some of which in presence of EDTA supported any assumption of metal induced DNA degradation. Fractions A1& C1 at 36˚C or 45˚C of Figure 3.8 showed that the λ-HindIII target DNA was mostly still intact after 45h of incubation whereas under the same conditions, A1 and B1 degraded λ-HindIII target DNA at 45˚C. The results justified ruling out the possibility of metal-induced-degradation.

4.2 Concentration and location

Observations supported by data obtained from the different experiments, suggested that the putative activities' of nuclease is slow most likely due to low concentration of the putative nuclease enzyme; it is expected that such stable enzymes that has a highly specific function are usually needed in small quantities depending on their biological function. In addition, the data indicated that soluble activity (A1) as well as envelope activity (B1) of *C. jejuni* can be discerned; Figure 5.8 illustrates that one nuclease activity (A1 fraction) required the higher temperature to activate. Fraction B1, on the other hand, was equally active at both 36 and 4˚C. Further investigations of the two entities need to be carried out before any solid conclusions can be made.

As indicated in Figure 3.8 experiments with fractions A1, B1, and C1 obtained from *C. jejuni*, the cytosolic fraction A1 showed poor activity relative to B1 fraction mostly made of cell envelope indicating that the nuclease activity was mostly associated with the cell envelope; the cytoplasmic membrane, cell wall, outer membrane, and the periplasmic compartment suggesting that the nuclease activity was mostly membrane bound or periplasmic in location that required SDS solubilization to release the activity. No experiments were conducted to test these two possibilities.

The Gel Retardation that may have been observed in Figure 3.5 also known as Electrophoretic mobility shift (Sambrook and Russell, 2006) where DNA complexed with a protein or another bulky molecule mobility is slowed down due to modified shape, electric charge, and or Molecular weight. Short DNA probes are more sensitive to retardation than large DNA molecules. The slow movement of PCR amplicon suggests that DNA was complexed with a relatively significant bulky molecule. This result may explain the smearing of PCR products obtained with all three bacteria; (*Helicobacter pylori*, *C. jejuni*, and QUBC70). The unique bright band seen in the ASt-45˚C/PCR lane (Figure 3.6) although may be explained by gel retardation, it is unlikely since it is too large and cannot be accurately explained.

4.3 Stability and optimal temperature

Only in two incidents that any lysate or preparation had failed to degrade any given DNA. First when the lysate was prepared in 10% SDS the activity may have been totally

obliterated. Another instant was observed when the bacterial culture (QUBC70) was heavily contaminated by another unknown bacterium. Otherwise, although inconclusive experiments (not shown) indicated that EDTA, 2-ME, pronase, proteanase K, 1% SDS, 2-ME, boiling for 10 min in alkaline SDS, phenol extraction all had failed to completely or significantly inhibit the putative activity of *C. jejuni*, QUBC 70, similar observations were made when the long procedure for DNA extraction was applied to *H. pylori* ATCC 43526. The nuclease activity was detected after lengthy incubation at >51ºC for 45h which resulted in the degradation of homologous DNA and slight degradation of λ-Hind III DNA (Figure 3.10). The results indicated that although the nuclease was heat stable and did not lose its activity even after 10 min boiling; yet its optimal temperature may be near 45 ºC possible 42 ºC which was not tested. However at 4˚C the activity was immensely reduced but retained for more than 4 weeks; storing fresh cell lysate at 4˚C allowed intrinsic DNA preservation and use as a control lysate (no treatment) as shown in Figure 3.4 lane 12 for QUBC 70.

4.4 Exonuclease or endonuclease

It is not clear whether the *C. jejuni* putative nuclease is a 5'→ to 3' or 3'→5' exonuclease or not. Although the PCR resistance (Figure 3.5) suggests that available 5'-sticky end of HindIII were susceptible to degradation; DNA with 3'-sticky end was not tested, but it is unlikely to be a better substrate than the 5'-sticky DNA since the 3'-end of blunt end is more available than the 3'-enfd of 5'-sticky HindIII-DNA, yet blunt end DNA appeared less susceptible than HindIII-DNA.

4.5 Nuclease of *C. jejuni* and QUBC70

Exogenous *E. coli* DNA which appeared as long streaks (Fig. 3.4; dsDNA lane 8 or ssDNA). Degradation of the *E. coli* DNA was not evident with 15%, 35%, or 50% AS fractions which had no activity (it was shown later that the QUBC70 activity was precipitated with AS at >180%. Possible *E. coli* DNA-methylation may be involved. The experiment illustrated in Figure 3.4 could not distinguish between ssDNA and dsDNA susceptibility to degradation simply because the source of DNA was not a suitable substrate (lane 11 vs. control lane 8).

The molecular weight of the putative nuclease from QUBC70 now named (NucQ70) appear to be consistent with that reported for NUC superfamily (SMART accession no. SM00477) characteristic for DNA/RNA nonspecific endonucleases (Gaasbeek et al., 2010). NucQ70 may fall in the region indicated by the arrows (Figure 4.12, panel II), this is supported by the unique presence of these bands in fractions (AS200 and 300%) which showed nuclease activity.

The precipitation of nuclease activity from *C. jejuni* lysate was affected by two variables; the AS saturation which varied with temperature and should have been carried out on ice rather than at room temperature due to its fluctuation depending on the time of the year (where it could reach 36 in hot Summer days). The other factor is the protein concentration in the *C. jejuni* lysate, it was observed that when OD600 was high, the activity could be precipitated at lower AS concentrations as seen in Figure 3.11 (lanes 25% and 50%) for *C. jejuni*. QUBC 70 was less affected since 2 and 3-fold AS were required.

4.6 Conclusion

The nucleases from these bacteria appear to have many features in common. Although they may have different biological functions, one possibility is that they are involved in DNA uptake and genetic transformation since this function is consistent with low concentration, exonuclease, and cell envelope location, or simply a scavenger enzyme that supplies nucleotides as nutrients, although this function dictates that a degradation enzyme should be highly efficient. It is recommended that preparation of DNA be done below 4 ^{o}C with mercaptoethanol. Further testing should be done with acid treatment, digestion with other proteases, and inhibition by nucleotide analogues as well as varying concentrations of SDS.

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نشاط إنزيم الحمض النووي في البكتيريا اللولبية

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ملخص:

يهدف هذا لبحث إلى دراسة نشاط وخصائص إنزيم تكسير الحمض النووي (deoxyribonuclease) في البكتيريا اللولبية مثل هليكوباكتر بيلوري، كامبيلوباكتر، "القدس 70"، حيث أن هذا النشاط الإنزيمي يتداخل عمله ويؤثر على تحضير الحمض النووي، عزل الحمض النووي، حفظه، ودراسة الحمض النووي بواسطة PCR ومعرفة تسلسله. من القواسم المشتركة بين هذه البكتيريا أنها بطيئة النمو، سالبيه غرام، لولبية الشكل. تكمن أهمية هذه البكتيريا بأن معدلات الإصابة بالهيلكوباكتر بيلوري قد تتجاوز 50% على مستوى العالم، و 85% عند الفلسطينيين ومثلها الكمبيلوباكتر، مما يجعل أهمية قصوى للبحث عن طرق للكشف عن هذه البكتيريا في عينات البراز والمياه والطعام وغيرها من العينات. الإنزيم تحت الدراسة يجعل الكشف عن هذه البكتيريا صعب للغاية بواسطة الطرق التي تعتمد على الحمض النووي وقد ركز هذا البحث على التعرف على هذا الإنزيم وصفاته وخصائصه وإمكانية منع نشاطه.

تمت دراسة نشاط هذا الإنزيم بطرق مختلفة وفي بكتيريا لولبية مختلفة وتحت ظروف مختلفة بعد زراعة البكتيريا وتكسيرها بطرق مختلفة. تمخض عن هذا البحث عدم إمكانية تثبيط نشاط هذا الإنزيم في هذه البكتيريا بعد تحليلها باستخدام المادة الكيماوية (SDS) والتي تذيب جدار البكتيريا وتفقد البروتينات شكلها وعملها حتى مع الغليان والذي يفقد الإنزيمات نشاطها بشكل عام ولكن عند تحليل هذه البكتيريا عن طريق استخدام الأمواج الصوتية وإضافة إنزيم اللايسوزيم الذي يحلل جدار الخلية تأثر نشاط إنزيم الحمض النووي ويمكن أن يعزى ذلك وجود الانزيمات الهاضمة للبروتينات أو أن انزيم Nuclease مرتبط بغلاف الخلية. إضافة كبريتات الأمونيوم المشبع (0.6 كبريتات الأمونيوم

إلى 1 ضعف الحجم البكتيري) عند درجة حرارة 25 مئوية إلى الكمبيلوباكتر جيجيني المحللة عمل على ترسيب الأنزيم مع الاحتفاظ بنشاطه، أما عندما كانت الكمية أقل من 0.6 فقد فشل المحلول في ترسيب الإنزيم المطلوب الدراسة. أما في حالة بكتيريا "القدس 70" استطاع محلول كبريتات الأمونيوم المشبع أن يرسبها قرب 300% (1:3 حجم كبريتات الأمونيوم المشبعة إلى حجم محلول البكتيريا). وبعد ذلك يتم خلط DNA- λ مع البكتيريا المحللة المراد فحص نشاط الانزيم فيها وحضنها على درجة حرارة 36 مئوية أو 45 لأزمان مختلفة تبين أن نشاط إنزيم الحمض النووي كان موجوداً في محلل بكتيريا الكمبيلوباكتر و"القدس "70 ولكن النشاط الإنزيمي تحت الدراسة الموجود في بكتيريا "القدس "70 كان مختلفاً عن ذلك الموجود في الكمبيلوباكتر بطريقة كبريتات الأمونيوم المشبعة، ونشاط الإنزيم في الكمبيلوباكتر كان على درجة حرارة 36 و 45 درجة مئوية ولكن ليس فاعلا على درجة حرارة 50 مئوية بوجود من SDS.