ABSTRACT

LEE, JAE YUN. Enzyme Properties and mRNA Expression of an NAD$^+$ Scavenging System: NatV and NadV of *Vibrio parahaemolyticus* Phage KVP40 (Under the direction of Eric S. Miller.)

KVP40 is a T4-like bacteriophage whose dsDNA genome (244,835 bp) has been sequenced (21). It infects *Vibrio parahaemolyticus*, which can cause disease in fish and shellfish, and gastroenteritis in humans when consumed in raw or under-cooked seafood (i.e., oysters). The KVP40 genome carries bacterial-like genes that have the potential to encode a pyridine nucleotide scavenging system for synthesis of NAD$^+$. This is the first pyridine nucleotide salvage pathway predicted from a phage or eukaryotic viral genome (21). *nadV* and *natV* are the two genes that encode the hypothetical two-reaction NAD$^+$ scavenging pathway. NadV, a nicotinamide phosphoribosyltransferase, catalyzes the first reaction that converts nicotinamide to nicotinamide mononucleotide (NMN). The NatV NMN adenyllyltransferase activity yields NAD$^+$. Nicotinamide adenine dinucleotide (NAD$^+$) is an essential cofactor involved in fundamental processes in cell metabolism, and pathways for its synthesis are potential anti-microbial, therapeutic targets. The phage enzymes provide model targets for these studies. NatV, which catalyzes the second step of the pathway, also has a Nudix hydrolase domain in the C-terminal half. The purpose of this project was to characterize expression patterns of *nadV* and *natV* during KVP40 development in *V. parahaemolyticus* by using qRT-PCR; to characterize the enzymatic activity of NadV and NatV in a coupled-enzyme assay using alcohol dehydrogenase (ADH) to connect to fluorescent NADH; to identify possible substrates for the NatV Nudix hydrolase; and to use mass spectrometry to
quantify product yields of the NatV reactions. qRT-PCR analysis showed that KVP40 nadV and natV were expressed early during infection relative to other phage genes used in this study. NadV-His6 and NatV-His6 enzymes were successfully purified by Ni²⁺ affinity HPLC and the levels of NADH produced were measured in a three step NadV-NatV-ADH reaction system. In the coupled assay, NatV-His6 converted NMN to 50 µmole of NAD⁺/sec/µg at 25°C. NatV NMNATase activity was also confirmed by mass spectrometry, in this assay, the rate was 350 µmole NAD⁺/sec/µg NatV-His6 at 25°C. NadV NAmPRTase activity was confirmed by producing 5.2, 4.9 and 5.0 µmole NMN/sec/µg NatV-His6 enzyme at 25, 30 and 37°C, respectively. Purified NadV used in the coupled enzyme also produced 2.7, 1.5, 1.3, 1.8 or 1.5 µmole NMN/sec/µg NadV-His6, when 10, 25, 40, 50 or 100 pmole NadV-His6 was respectively used in the reaction at 25°C. The “Nudix” activity of purified NatV was measured by a phosphate releasing assay and by mass spectrometry. Using the phosphate release Nudix hydrolase assay, ADP-ribose was identified as a preferred substrate for KVP40 NatV, followed by NAD⁺, NADH, and NADPH. In this assay, ADP-ribose as substrate yielded 0.6 µmole phosphate/sec/µg NatV-His6 at 37°C, when Mg²⁺ was supplied as the required metal ion. Mass spectrometry verified the NatV Nudix hydrolase preference for ADP-ribose as substrate, yielding the same rate of 0.6 µmole AMP/sec/µg NatV-His6 at 37°C in the presence of Mg²⁺. Together these data confirm the various enzymatic activities of key pyridine nucleotide scavenging enzymes encoded by phage KVP40. The time course of expression and in vivo activities suggest that pyridine nucleotide scavenging during KVP40 infection of V. parahaemolyticus is a functional and potential relevant pathway used by the
phage.
Enzyme Properties and mRNA Expression of an NAD$^+$ Scavenging System: NatV and NadV of Vibrio parahaemolyticus Phage KVP40

by

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2008

APPROVED BY:

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                                             Chair of Advisory Committee
DEDICATION

This thesis is dedicated to my parents, Ik Boum Lee and Myoung Sook Yun, and to my brother, Jae Seung, my guardian in heaven. I thank them for their support and unconditional love throughout the course of this thesis.
BIOGRAPHY

Jae Yun Lee was born on February 1st, 1979, in Seoul, South Korea. Until she moved to the United States to experience intensive English practice, she was a college student, majoring in life science at Suwon University. When she audited a biology class for an assignment in English as a Second Language (ESL) program, she was fascinated by the student-involved lecture style in American colleges. Later, she transferred and received a Bachelor of Science in Microbiology from North Carolina State University in 2004. While she attended NCSU, she joined the Microbiology Club, the Asian Student Association (ASA), and the Korean Student Association (KSA) and was involved in many volunteer services. During her employment as a research technician at the USDA’s Agricultural Research Service, working on nitrogen fixation of Cyanobacteria, she decided to pursue her skills and learn more of microbiology in depth. Therefore, she made a decision to continue her studies in the NCSU graduate program in Microbiology. After graduation, she plans to go back to South Korea.
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There are a number of people I would like to thank for making this study possible:

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- Dilan Weerakoon (French Press): Microbiology, NCSU
- Alice Lee (HPLC): Microbiology, NCSU
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I. LITERATURE REVIEW

1.1 Overview

Lytic T4-like bacteriophage KVP40 was isolated from polluted seawater off the coast of Japan. Its hosts include *V. parahaemolyticus*, *V. cholerae*, and non-pathogenic *Vibrio* species such as *V. natriegens*. Its genome has been sequenced and revealed about 400 genes, with several “core” genes resembling T4. Hypothetical KVP40 genes are not in the database. Other genes were previously only seen in bacteria, including “pyridine nucleotide metabolism genes” (21).

Each year, approximately 4,500 cases of *Vibrio parahaemolyticus* infection, a leading cause of gastroenteritis from undercooked seafood, occur in the United States (27) (http://www.cdc.gov/nczved/dfbmd/disease_living/vibriop_gi/html#1). This bacterium can also cause disease in fish and shellfish.

Phage were discovered by Twort, and by d’Hérelle, in 1915 – 1917 (4) and were originally proposed as “lytic” therapeutic agents. Antibiotics were later discovered and used almost exclusively as antimicrobial. However, in an era of antibiotics resistance, phages are of renewed interest. They may be used as therapeutic agents, as a source of medical or agricultural reagents, for new molecular biology enzymes, and for basic biochemical and genomic studies. Several other T4-type bacteriophage genomes have now been sequenced (www.phage.bioc.tulan.edu).

Analysis of KVP40 and its genome sequence will provide new biological insights and potential new diagnostic or therapeutic reagents for treating *Vibrio* infections. In this
thesis, I focus on the properties and expression patterns of the predicted pyridine nucleotide scavenging pathway encoded by the KVP40 $nadV$ and $natV$ genes.

1.2 Bacteriophage KVP40

T4-like bacteriophage KVP40 belongs to the Myoviridae family of phages that have prolate icosahedral heads and contractile tails. It is lytic and has a broad-host-range, including Vibrio parahaemolyticus, Vibrio cholerae and other Vibrio spp. KVP40 has a linear, circularly permuted chromosome of dsDNA (24) (21). The genome is 244,835 bp and 42.6 % G+C (Figure 1) (21). Although KVP40 shares many “core” features with T4 bacteriophage, there are several differences: KVP40 has a longer head and a larger genome; it lacks several T4 enzymes that are involved in host DNA degradation and group I intron activities; and it lacks the middle-mode transcription activator protein MotA. The KVP40 genome consists of 386 predicted protein coding sequence (CDSs), although the function of 65 % of the CDSs is unknown. It also contains 30 tRNAs, 33 predicted T4-like late promoters, and 57 predicted rho-independent transcription terminators. Overall, 92.1 % of the KVP40 genome codes for protein and at least 99 CDSs are homologues to CDSs of T4. When the genome sequence was reported, some KVP40 CDSs had homologs only in cellular organisms (not virus). Table 1 lists genes of KVP40 that were identified as pyridine nucleotide metabolism-related. Included in this list are $nadV$, $natV$ and others that were proposed to be engaged in a NAD$^+$ scavenging pathway, the first noted incidence of such genes in a viral genome (21). $nadV$ and $natV$ have more recently been seen in other double-stranded phages, including E. coli phage...
RB43 and *Aeromonas* phages Aeh1, 31, 44RR2.8t, and 25 (Table 2). In a possible two-reaction NAD$^+$ scavenging pathway, *nadV* encodes nicotinamide phosphoribosyltransferase (NAmPRTase) and *natV* encodes NMN adenylyltransferase (NMNATase), catalyzing NMN to nicotinamide dinucleotide (NAD$^+$). The diversity of the NAD$^+$ biosynthesis pathway in bacteria is described in section 1.5 below.
Figure 1. KVP40 CDS map (21).

- Predicted coding sequences: 386
- T4-like genes (26%): 99
- Related to genes of cellular genomes (8%): 26
- Unique, hypothetical (65%): 253
- tRNAs and 5 “pseudo” tRNA genes: 25
- Host-like “Nad” genes (10%):
**Table 1.** KVP40 NAD⁺-related CDS homologues found in other organisms (21).

<table>
<thead>
<tr>
<th>KVP40 CDS (aa)²</th>
<th>Homolog (aa)²</th>
<th>Description</th>
<th>Organism</th>
<th>No. Of residues/total (%)</th>
<th>E value</th>
<th>Identical</th>
<th>Similar</th>
</tr>
</thead>
<tbody>
<tr>
<td>264 (497)</td>
<td>NadV (495)</td>
<td>NamPRTase</td>
<td>Haemophilus</td>
<td>32</td>
<td>48</td>
<td>2e-53</td>
<td></td>
</tr>
<tr>
<td>162 (341)</td>
<td>NADM_SYNY3 (339)</td>
<td>NMNATase/Nudix</td>
<td>Synechocystis</td>
<td>33</td>
<td>51</td>
<td>3e-44</td>
<td></td>
</tr>
<tr>
<td>043 (240)</td>
<td>Sir2 (234)</td>
<td>NAD hydrolysis</td>
<td>Helicobacter</td>
<td>39</td>
<td>57</td>
<td>1e-28</td>
<td></td>
</tr>
<tr>
<td>215 (221)</td>
<td>PnuC (241)</td>
<td>NMN transport</td>
<td>Yersinia</td>
<td>31</td>
<td>47</td>
<td>2e-19</td>
<td></td>
</tr>
<tr>
<td>211 (326)</td>
<td>NadR (323)</td>
<td>NMN transport, NMN-ATase, Transcriptional regulator</td>
<td>Mycobacterium</td>
<td>26</td>
<td>45</td>
<td>1e-13</td>
<td></td>
</tr>
</tbody>
</table>

² CDS number and length of encoded protein in amino acid (aa).
² Proteins are listed by name or GenBank locus, with the length given.

**Table 2.** Other large dsDNA phages with nadV and natV in their respective genomes (GenBank; Also see http://phage.bioc.tulane.edu/)

<table>
<thead>
<tr>
<th>Phage &amp; Host</th>
<th>NadV</th>
<th>NatV</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>KVP40 Vibrio</strong></td>
<td>497 / 0.0</td>
<td>341 / 0.0</td>
</tr>
<tr>
<td><strong>Aeh1 Aeromonas</strong></td>
<td>481 / 2e-113</td>
<td>351 / 9e-55</td>
</tr>
<tr>
<td><strong>RB43 E. coli</strong></td>
<td>476 / 3e-111</td>
<td>352 / 7e-64</td>
</tr>
<tr>
<td><strong>31 Aeromonas</strong></td>
<td>484 / 8e-106</td>
<td>352 / 7e-47</td>
</tr>
<tr>
<td><strong>44RR2.8t Aeromonas</strong></td>
<td>484 / 2e-105</td>
<td>352 / 1e-46</td>
</tr>
<tr>
<td><strong>25 Aeromonas</strong></td>
<td>493 / 1e-103</td>
<td>352 / 2e-50</td>
</tr>
<tr>
<td><strong>K Staphylococcus</strong></td>
<td>489 / 7e-54</td>
<td>-</td>
</tr>
<tr>
<td><strong>G1 Staphylococcus</strong></td>
<td>454 / 5e-53</td>
<td>-</td>
</tr>
<tr>
<td><strong>P100 Listeria</strong></td>
<td>597 / 7e-43</td>
<td>-</td>
</tr>
<tr>
<td><strong>Felix 01 Salmonella</strong></td>
<td>593 / 83-29</td>
<td>-</td>
</tr>
</tbody>
</table>
1.3 *Vibrio parahaemolyticus*

In this study, the bacterial host cell infected by phage KVP40 is *Vibrio parahaemolyticus* (lab strain EM343). *V. parahaemolyticus* is a lytic, halophilic, Gram-negative marine bacterium (39). It can cause disease in fish and shellfish and can cause food-borne gastroenteritis in humans when consumed with raw or improperly-cooked seafood. The generation time of *V. parahaemolyticus* can be extremely short and it has various lifestyles such as a planktonic, free-swimming form, and as a biofilm on the bottom of boats or on the surface of objects or organisms in the ocean. Its whole genome sequence (Asian pandemic strain RIMD 2210633, isolated from a patient with travelers’ diarrhea at the Kansai Intl’ Airport quarantine station in 1996) is available at NCBI. It has two circular chromosomes of 3,288,558 bp and 1,877,212 bp (Table 3). Chromosome 1 contains genes that are involved in cell growth and viability, whereas chromosome 2 has genes that are engaged in cell metabolism, transcriptional regulation and substrate transport. *V. parahaemolyticus* also possesses genes on chromosome 2 for a type III secretion system (TTSS), a critical virulence factor of diarrhea-causing bacteria such as *Salmonella* and *Shigella*, and is important for *Yersinia* and plant pathogens. *V. cholerae* lacks this factor (17). The needle-like TTSS can directly inject bacterial proteins into host cells. (17). Although the thermostable direct haemolysin (TDH) and TDH-related haemolysin (TRH) are two additional virulence factors, these are not strictly involved in human pathogenesis by *V. parahaemolyticus* (5).
Table 3. Summary genome features of *V. parahaemolyticus* (17)

<table>
<thead>
<tr>
<th></th>
<th>Chromosome 1</th>
<th>Chromosome 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length of sequence (bp)</td>
<td>3,288,558</td>
<td>1,877,212</td>
</tr>
<tr>
<td>G+C ration</td>
<td>45.4 %</td>
<td>45.4 %</td>
</tr>
<tr>
<td>Number of coding sequence</td>
<td>3080</td>
<td>1752</td>
</tr>
<tr>
<td>Protein coding region</td>
<td>86.9 %</td>
<td>86.9 %</td>
</tr>
<tr>
<td>Average length of coding sequence (bp)</td>
<td>926.9</td>
<td>931.3</td>
</tr>
<tr>
<td>Hypothetical proteins</td>
<td>1090 (35 %)</td>
<td>756 (43 %)</td>
</tr>
<tr>
<td>rRNA operons</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>rRNA</td>
<td>112</td>
<td>14</td>
</tr>
</tbody>
</table>

1.4 Importance of nicotinamide adenine dinucleotide in living cells

The pyridine nucleotide pool, composed of NAD$^+$ and its derivatives NADH, NADP$^+$ and NADPH, is essential in all living organisms (11). As a coenzyme, NAD$^+$ is involved in innumerable oxidation-reduction, electron transfer and energy controlling reactions; nutrient and metabolic processing; as a substrate in covalent ADP-ribosylation of protein; as a substrate for NAD-dependent bacterial DNA ligases in DNA replication, recombination and repair; and in protein deacetylation by the CobB/Sir2 family of enzymes, often as an allosteric effector to change or inactivate enzyme activity (42) (11) (35) (7) (38). The essential uses of pyridine nucleotides recommend them as attractive therapeutic targets for potential antimicrobial compounds (3).
1.5 NAD$^+$ synthesizing pathways

Studies on NAD$^+$ biosynthesis have revealed that NAD$^+$ is made from nicotinamide or nicotinate, which are the so called ‘niacins’. Niacin was first identified as a compound that prevents pellagra in dogs that lack dietary tryptophan, one of the precursors in $de$ $novo$ biosynthesis of NAD$^+$ (25). There are two major routes to NAD$^+$ production in living cells: a $de$ $novo$ pathway and a salvage pathway (Figure 2). In bacteria, when there is an accessible exogenous source of the pyridine ring, endogenous $de$ $novo$ synthesis is suppressed and scavenging is preferred (14).
Figure 2. Overview of NAD biosynthesis and salvage pathways in different bacteria. Abbreviations: quinolinate synthase (nadA), quinolinate phosphoribosyltransferase (nadC), nicotinate mononucleotide adenyllytransferase (nadD), NAD synthetase (nadE), niacin transporter (niaP), nicotinamide deaminase (pncA), nicotinate phosphoribosyltransferase (pncB), nicotinamide phosphoribosyltransferase (nadV), nicotinamide mononucleotide adenyllytransferase (nadM\textsuperscript{AT}), nicotinamide riboside kinase (nadRK), ribose phosphate pyrophosphokinase (prs), Asp, aspartate; Trp, tryptophan; IA, iminoaspartate; Qa, quinolinic acid; NaMN, nicotinate mononucleotide; NaAD, nicotinate adenine dinucleotide; NA, nicotinic acid; Nam, nicotinamide; RNam, ribosynicotinamide, NMN, nicotinamide mononucleotide; ADPR, ADP-ribose; PRPP, phosphoribosyl pyrophosphate; Rib-P, ribose-5-phosphate; Xyl-P, xylulose-5-phosphate; Ara, L-arabinose; Xyl, D-xylulose; PNC IV, four-step pyridine nucleotide cycle; PNC VI, six-step pyridine nucleotide cycle (36).
1.5.1 De novo pathway

Most organisms, including *Escherichia coli* and *Salmonella enterica*, generate NAD\(^+\) through a *de novo* pathway (19). In the *Salmonella de novo* pathway, quinolinic acid (QA) is formed from L-aspartate and dihydroxyacetone phosphate (DHAP) via the *nadB* (L-aspartate oxidase) and *nadA* (quinolinic acid synthetase) enzymes (13) (45) (1). QA coupled with 5-phosphoribosyl-1-pyrophosphate (PRPP) is converted to nicotinic acid mononucleotide (NaMN) and then to nicotinic acid adenine dinucleotide (NaAD). In an alternative way, NaMN can be generated from nicotinic acid (via *pncB*; nicotinic acid phosphoribosyltransferase) that is either directly transported from outside of the cell or converted from exogenous nicotinamide with cooperation of *pncA* (nicotinamide deaminase). NAD\(^+\) and NADP\(^+\) can be formed from NaAD\(^+\) by amidation of OH to NH\(_2\). Since nicotinic acid and nicotinamide only differ by the one amide group, it was predicted that both can serve as precursors in the *de novo* pathway.

*nadR* encodes an NMNAT activity. Moreover, it was originally described as a transcriptional regulator that represses genes involved in NAD\(^+\) biosynthesis and pyridine nucleotide salvage in *Salmonella typhimurium* (19) (32) (9) (10) (45). When NadR is bound to the corepressor, NAD\(^+\), transcription of *nadA*, *nadB* and *pncB* are repressed (8) (41) (30). NadR of *S. typhimurium* possesses a helix-turn-helix DNA binding domain, which does not exist in the NadR homologue of *H. influenzae*; there is no transcriptional repression system observed in this organism. However, NadR of *H. influenzae* was discovered to have a nicotinamide ribonucleoside kinase (RNK) domain located in the C-terminus, whereas the NMNAT motif is located in the N-terminus (39). This was confirmed by structural and
biochemical analysis (19). The RNK domain consists of a Walker A (P-loop) domain, responsible for ATP binding, and a Walker B domain, responsible for ATP hydrolysis. As NadR displays multifunctional activities, including enzymatic, transport, and DNA binding, the protein plays an important role in NAD$^+$ synthesis and control of the NAD$^+$ de novo biosynthesis pathway (19).

1.5.2 Salvage pathway

Some bacteria do not have the ability to form NAD$^+$ via de novo synthesis; they reuse NAD$^+$ via a pyridine nucleotide salvage pathway. *Salmonella typhimurium* can also generate NAD$^+$ break-down products through pyridine nucleotide cycle enzymes (PNC) that recycle several byproducts or intermediates (8). In the enteric PNC system, nicotinic acid (NA), nicotinamide (Nam) or the pyridine mononucleotides (NaMN/NMN) from either environmental or internally recycled sources can be converted to NMN and to NAD$^+$ by *pnc/pnu* gene products. The functional pyridine nucleotide cycle pathway in *S. typhimurium* was initially demonstrated by Foster and Baskowsky-Foster (9). NAD$^+$ can be recycled via two PNC pathways, PNC IV, which refers to a four-step cycle, and PNC VI, which refers to six-step cycle (8). PNC IV plays a major role in the intracellular NAD$^+$ recycling pathway of *S. typhimurium* due to the amount of recycled intracellular NAD$^+$, 60-69% via PNC IV and 31-40% via PNC VI (8). The degraded compounds from NAD$^+$ can be used again to synthesize NAD$^+$ in the presence of ATP (8).
1.6 V factor dependent Pasteurellacea

In many bacteria, NAD⁺ synthesis can occur through either a de novo synthesis via quinolinic acid or a pyridine nucleotide salvage pathway. However, members of the bacterial family Pasteurellaceae do not have either a full de novo or complete PNC pathway to synthesize NAD⁺, so these bacteria must obtain NAD⁺ from the environment as NAD⁺ itself or from only few precursors, including NMN and nicotinamide riboside (NR). These three compounds are known as V factors and many Pasteurellaceae members are V factor dependent (Figure 3A) (18) (23).
Nicotinamide Adenine Dinucleotide (NAD$^+$)

Nicotinamide Mononucleotide (NMN)

Nicotinamide Riboside (NR)

A. V factors NAD$^+$, NMN and NR have aminated pyridine-carbonyl groups and a pyridine-ribose bond.
B. Non-V factor pyridine Quinolinate, NaMN, NA and Nam.

**Figure 3.** Structure of V factors (A) and Non-V factors (B) of Pyridine Nucleotide Metabolism (18). Only V factors (A) support growth of strains lacking *de novo* or salvage pathways.
1.6.1 Identification of NadV (NAmPRTase) in *Haemophilus spp.*

Generally, *Haemophilus* spp., including *Haemophilus haemoglobinophilus*, are known as V factor dependent *Pasteurellaceae*, as these show a requirement for NAD$^+$ (15). However, there are a few exceptions. Some species of *Pasteurellaceae*, including *Actinobacillus pleuropneumoniae*, can grow without V factor (12). *Haemophilus paragallinarum*, *Haemophilus parainfluenzae*, and *Haemophilus ducreyi*, are V factor independent (12) (18). These species are capable of synthesizing NAD$^+$ from nicotinamide by the activity of two enzymes: nicotinamide phosphoribosyltransferase, which converts nicotinamide (Nam, Figure 3B) to NMN, and NMN adenylyltransferase, which catalyzes NMN to NAD$^+$ (Figure 4). The V factor independence gene, *nadV*, is encoded on a plasmid, pNAD1 in three *Haemophilus* species, while *nadV* is present on the chromosome of *H. haemophilus* (18) (26) (12). The 5568 bp circular plasmid pNAD1 contains *nadV* (nicotinamide phosphoribosyltransferase) (Figure 5) and other genes that suggest a phage origin for at least some of pNAD1 (23).
Figure 4. The two-reaction pathway for biosynthesis of NAD$^+$ as found in the V-factor independent family Pasteurellaceae. Nicotinamide is scavenged directly into NAD$^+$ (18).
Figure 5. Map of pNAD1 from *Haemophilus paragallinarum, Haemophilus parainfluenzae*, and *Haemophilus ducreyi*, containing the *nadV* nicotinamide phosphoribosyltransferase gene. (23) *plp* genes encode “phage-like” proteins.

1.6.2 Homologues of NadV (NAmPRTase)

The NadV enzyme was originally identified as a pre-B cell colony-enhancing factor (PBEF), a cytokine that is secreted to stimulate B cell development (37) (16) (34). PBEF has been shown to have nicotinamide phosphoribosyltransferase (NamPRTase) activity (34). Unfortunately, many NadV-type NamPRTases in sequenced bacterial genomes are annotated as “PBEF” proteins. Recently, the PBEF enzyme was also identified as a visceral fat-derived hormone and was called “visfatin”. The function of visfatin is to bind insulin receptor (IR) and thereby mimic the action of insulin in mammalian cells (34) (22). NadV as a nicotinamide phosphoribosyltransferase in NAD biosynthesis has an apparent ancient origin and exhibits high homology between bacteriophage, bacteria, and vertebrates (Figure 6) (34) (18).
Figure 6. Alignment of NadV proteins. Each is a member of the conserved domain pfam04095.1 (NAPRTase) and COG1488.1. Highly homologous regions are boxed and identical residues are in red. The enzyme activity in *H. ducreyi* has been characterized. The *His*-238 region (*) is similar to the phosphohistidine site of *Salmonella* NAPRTase (34).
1.6.3 NadV and a two-reaction pyridine nucleotide scavenging pathway of phage KVP40

The complete genome sequence of KVP40 revealed a 1494 bp *nadV* gene. Alignment of NadV proteins showed substantial degree of conservation of the domain, including a phospho-histidine site used for energy coupling. Identification of other genes (Table 1) in the genome sequence led to a proposed pyridine nucleotide salvage pathway encoded by phage KVP40. This resembles the two-step *Haemophilus* type reactions: nicotinamide obtained from the environment is converted to nicotinamide mononucleotide (NMN) by nicotinamide phosphoribosyltransferase (*nadV*) and then a NMN adenylyltransferase (NMNATase) catalyzes conversion of NMN to nicotinamide dinucleotide (NAD\(^+\)) (Figure 7). NadV is the key first reaction and two KVP40 proteins NatV and NadR have predicted NMNATase activity.
Figure 7. Hypothesized pyridine nucleotide salvage pathway encoded by KVP40. The components of the NAD salvage pathway (in solid lines), which are mostly related to bacterial enzymes, have not previously identified in phages. The PnuC/NadR complex transports nicotinamide mononucleotide (NMN) across the bacterial membrane (hatched bar) in Salmonella. NadV, nicotinamide phosphoribosyltransferase (NamPRT) which converts nicotinamide to NMN, resembles the enzyme in Haemophilus ducreyi. NadR is bifunctional with NMN adenylyltransferase (NMNAT) activity and its domain is similar to that of bacterial enzymes. Nudix hydrolase is highly homologous to the enzyme found in the marine cyanobacterial species Synechocystis. Hydrolysis of NAD/NADH (dashed lines) can be catalyzed by the NatV Nudix hydrolase and by Sir2/CobB (also not previously found in phages) (21).

1.7 NMNATase: the second reaction in a pyridine nucleotide scavenging pathway

NAD$^+$ can be directly formed from NMN via the reaction catalyzed by NMN adenylyltransferase (NMNATase), whereby the adenylyl moiety from ATP is transferred to NMN (44) (30). NMN can be either transported from the environment or recycled from NAD$^+$ intracellularly (45). NAD$^+$ also can also be derived from nicotinate mononucleotide (NaMN) (catalyzed by NMNATase) to form nicotinate adenine dinucleotide (NaAD), followed by addition of an amide group to form NAD$^+$ (catalyzed by NAD synthetase) (44).
1.7.1 Identification and homologues of NatV NMNATase

The KVP40 CDS designated as natV encodes a protein having an NMNATase domain (21). The NatV protein of KVP40 contains 339 amino acid residues: the N-terminal half is a predicted biosynthetic NMN adenylyltransferase, and its C-terminal half is a potential Nudix hydrolase (Figure 8). When archaeal NMN adenylyltransferase was found in *Methanococcus jannaschii* and the thermophilic archaean *Sulfolobus solfataricus*, it aligned with bacterial NMNATase of *E. coli* and *S. typhimurium* (NadR). *M. jannaschii* NMNATase has 29 residues at the N-terminus that are homologous with the enteric NadR (30). Also the N-terminal region of *Synechocystis* sp. slr0787 whose NMNATase is associated with a Nudix hydrolase domain at the C-terminal region, was compared and showed high homology to *M. jannaschii* NMNAT (30) (31) (13). The Nudix hydrolase activity in the C-terminal region of NatV in *Synechocystis* sp. NMNATase (slr0787) is discussed later in this chapter.
Figure 8. Bifunctional KVP40 NatV: \text{NMN adenyllytransferase (region:4-181)} / \text{Nudix hydrolase (region:207-324)}.

### 1.8 Nudix hydrolase: a possible second function of KVP40 NatV

The KVP40 NatV enzyme catalyzing the second step of the NAD$^+$ scavenging pathway possesses another possible activity called ‘Nudix’ hydrolase. The ubiquity of Nudix hydrolases and the highly conserved amino acid sequence at the KVP40 NatV C-terminus suggested a possible second function of KVP40 NatV as a Nudix hydrolase.

#### 1.8.1 C-terminal NatV: Nudix hydrolase domain

The ‘Nudix’ hydrolase superfamily consists of a large subfamily of proteins that has a highly conserved amino acid sequence domain, $\text{GX}_5\text{EX}_7\text{REUXEEXGU}$, where U is usually
hydrophobic amino acids Ile, Leu, or Val and X can be any amino acid (20) (29) (43). The family of enzymes is ubiquitous and thus found in archaea, eukaryotes, and prokaryotes. According to BLAST searches of Nudix consensus sequences (May, 2008), there are more than 800 “Nudix-like” open reading frames from more than 250 species. The Nudix box has a novel structure of loop-helix-loop motif which can play a fundamental role in substrate binding and catalysis. This enzyme is capable of cleaving substrates with an X-linked nucleoside diphosphate, where X can be a phosphate, a sugar, a nucleoside mono/diphosphate, etc (29). Because the nucleoside diphosphate linkage is a common recognition feature of Nudix hydrolases, the enzymes have a wide range of substrates, including NADH, dinucleotide polyphosphates, nucleotide sugars, cell signaling molecules and (deoxy) ribonucleoside triphosphates (33) (43). The roles of Nudix hydrolases in cell metabolism are involved in managing the cellular accumulation of potentially toxic small molecule compounds, which could be lethal for the cell, and in the regulation of intracellular levels of nucleotide cofactors and signaling molecules for cell growth and survival (33) (6) (29). *E. coli* MutT is a protein studied the most in the Nudix superfamily. *mutT*-defective mutants cause from 100- to 10,000-fold increase in A:T to C:G or G:C to T:A transversions. This can occur by either A:T → A~G or C~T → C:G mispairing during DNA replication (2) (28). The MutT enzyme hydrolyzes and removes mutagenic 8-oxo-dGTP from the DNA precursor pool.
1.8.2 Homologues of Nudix hydrolase

*Synechocystis* sp. slr0787 NadM (from strain PCC 6803) is a bifunctional enzyme, with nicotinamide mononucleotide adenylyltransferase and ‘Nudix’ hydrolase activity. The biochemistry of NadM has been well studied (29) (32) (13) (Figure 9). This enzyme from *Synechocystis* sp. is the only characterized bifunctional member of the bacterial Nudix superfamily (13). In one study, the T4 bacteriophage gene e.l (*nudE.1*), whose sequence is highly homologous to MutT, was registered as a new member of the Nudix family that can hydrolyze FAD, ADP-ribose, and AP₃A. It was the first gene from a bacteriophage that was cloned, expressed and characterized as a member of the ‘Nudix’ hydrolase superfamily (43). However, it is not bifunctional and only has the Nudix activity. Many studies have revealed that Nudix hydrolase activity, and NMN adenylyltransferase activity, require divalent metal ions, including either Mn²⁺, Mg²⁺ or Zn²⁺. (29) (31) (13). Alignment of KVP40 NatV, both N- and C-termini, with the *Synechocystis* bifunctional enzyme is presented in Figure 10.
Figure 9. Structure of Synechocystis NadM-Nudix (A) Bifunctional Synechocystis NadM monomer with both NMNATase and ADPRase (“Nudix”) domains. (B) Dimer of Synechocystis NadM (13).
1.9 Conclusion

The pyridine nucleotide salvage pathway is a critical metabolic pathway to synthesize NAD⁺ in many living cells that has not been previously characterized in phages or other viruses. In KVP40, the pathway is encoded by two proteins, NadV and NatV that catalyze reactions consecutively. NadV was originally identified as human PBEF which has NAmPRTase activity and the enzyme is highly homologous to NadV protein in H. ducreyi. NatV enzyme is similar to NadM in *Synechocystis* whose enzyme is bifunctional, having an NMNATase domain at the N-terminal region and a Nudix hydrolase domain in the C-
terminal region. Current experiments on KVP40 \(nadV\) and \(natV\) were initiated to disclose the pyridine nucleotide (NAD\(^+\)) salvage pathway in KVP40: the transcription patterns of two main genes, \(nadV\) and \(natV\), during phage development in \(V. parahaemolyticus\), and the function of the two enzymes NadV (NamPRTase) and NatV (NMNATase) in the reaction. The second role of NatV, Nudix hydrolase, was also investigated.
II. REFERENCES FOR LITERATURE REVIEW


Biochemistry. 47:1608-1621.


III. EXPERIMENTAL WORK OF THESIS

3.1 Introduction

The complete genome sequence of bacteriophage KVP40 revealed that it encodes a possible NAD\(^+\) scavenging pathway. This is the first pyridine nucleotide salvage pathway predicted from a phage or eukaryotic viral genome (15). Two genes, \textit{nadV} and \textit{natV}, would suffice to encode the hypothetical two-reaction NAD\(^+\) scavenging pathway. NadV is a nicotinamide phosphoribosyltransferase that converts nicotinamide to nicotinamide mononucleotide (NMN). NatV is a predicted nicotinamide mononucleotide adenylyltransferase that would catalyze conversion of NMN to NAD\(^+\). NAD\(^+\) is an essential compound in all living cells, as it is involved in cellular redox control, energy level and nutrient processing; serves as a substrate for ADP-ribosylation of protein; as a substrate for NAD-dependent bacterial DNA ligase in DNA replication, recombination and repair; in protein deacetylation catalyzed by CobB/Sir2 family (7) (25) (22). In the current projects, qRT-PCR analysis was used to disclose the expression patterns of \textit{nadV} and \textit{natV} relevant to other phage genes during infection. KVP40 \textit{nadV} and \textit{natV} were previously cloned into pET101/D-TOPO and pSMART HK80/90, respectively, and used here in for enzyme overproduction and purification. Purified His6-tagged NadV and His6-tagged NatV were used in a coupled-enzyme assay to confirm their enzymatic activities in the pyridine nucleotide scavenging pathway. NatV, a two-domain protein, possesses a second function in its C-terminal half, called Nudix hydrolase. The possible substrates for NatV as a Nudix hydrolase were investigated. The two activities of purified NatV were confirmed by mass spectrometry of enzyme reaction products.
3.2 Materials and Methods

3.2.1 NatV and NadV Characterization

3.2.1.1 NatV Expression and Purification

*natV* was previously cloned into pSMART BLUNT HK80 and HK90 vectors (Lucigen, MI, USA) by Zhiqun Li (M.S. Thesis, Dept. Microbiology, 2004) (11). These created C-terminal His6-tagged NatV proteins. Plasmid pZL166 (*natV* in pSMART-HK80) and pZL176 (*natV* in pSMART-HK90) were transformed into chemically competent BL21-AI cells (Invitrogen, CA, USA). Overnight grown BL21-AI/pZL166 (EM441) and BL21-AI/pZL176 (EM434) cells were diluted to OD$_{600}$ = ~0.1 into 16 ml of fresh LB medium containing kanamycin (50 μg/ml), tetracycline (12.5 μg/ml) and 20% D (+)-glucose (0.02% at final concentration). When cultures reached an OD$_{600}$ at ~0.5, 8 ml of each culture was taken as uninduced control. By adding 1 mM IPTG and 0.2% L-Arabinose, the remaining culture was induced and grown for an extra 4 hours. Cell pellets were collected by centrifugation at 5000 rev/min, for 10 minutes at 4°C. Cell pellets were stored at -20°C until disrupted. One of the induced NatV samples (BL21-AI/pZL166) was initially tested on Ni-NTA beads to confirm the affinity to nickel. The pellet was resuspended in 8 ml of binding buffer then lysed with a French Press 3 times at 1260 psi. About 8 ml of supernatant was collected by centrifugation at 5000 rev/min, 4°C for 15 minutes, and then passed through a 0.20 μm 26 mm syringe filter (Corning®, NY USA). The sample was loaded onto a 5 ml HPLC Ni$^{2+}$ column (GE healthcare, NJ, USA), which was previously charged with 0.1 M NiCl$_2$, in binding buffer A. A total of 41 fractions were collected at a flow rate of 1 ml/min for the linear gradient (first 35 ml) and 2 ml/min for isocratic flow with 100% buffer B (50
mM Na$_2$HPO$_4$, pH8.0, 300 mM NaCl, 500 mM imidazole) (protocol of Li: Nad purification, method 2.4-Jae NatV purification 3_25_08, Duo Flow HPLC, Grunden Lab, Microbiology dept, NCSU). Samples were analyzed on 12.5 % SDS-PAGE. Fractions 24, 25 and 26; 27, 28, 29, 30 and 31; 32, 33, 34 & 35 were combined and dialyzed overnight at 4°C in buffer (1 mM Tris-HCl, pH 5.0, 1 mM MgCl$_2$, 1 mM EDTA, 1 mM DTT and 20 % of glycerol), using a Slide-A-Lyzer 10 kDa MWCO dialysis cassettes (PIERCE, Rockford, IL, USA). Each sample recovered ~ 1 ml and 200 µl aliquots in 20% glycerol were stored at -80°C.

### 3.2.1.2 NadV Expression and Purification

The nadV gene was previously cloned from KVP40 by Z. Li, using pET101/D-TOPO (Invitrogen, CA, USA) (11). This created a C-terminal His6-tagged NadV protein. Briefly, chemically competent TOP10 cells (Invitrogen, CA, USA) were used to carry and propagate nadV plasmid pZL405 (EM424). Then, plasmid pZL405 was purified from EM424 using Qiagen Mini-prep kits (QIAGEN, CA, USA). NadV protein was expressed and purified by the protocol of Z. Li (M.S. Thesis, NCSU Microbiology 2004) (11). Plasmid pZL405 DNA was transformed into chemically competent BL21-AI cells and grown overnight at 37°C in Luria-Bertani medium with ampicillin (100 µg/ml) and tetracycline (12.5 µg/ml). The overnight grown culture was diluted into fresh 10 ml medium with a starting OD$_{600}$ of ~0.1. The 10 ml culture was grown until the OD$_{600}$ reached ~0.5 at 37°C (water bath shaker, 250 rpm). 5 ml of culture was removed as an un-induced control. By
adding 1 mM IPTG and 0.2% L-Arabinose, the remaining 5 ml culture was induced and grown for 4 hours. Cell pellets were collected by centrifugation at 5000 rev/min, 4°C for 10 minutes in a Sorvall® RC-5B Refrigerated Superspeed Centrifuge (Du Pont Instruments). Induced cell pellets (5 ml induced cultures, stored at -20°C) were suspended in 8 ml of binding buffer A (50 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 8.0, 300 mM NaCl), then disrupted 3 times by a French Press at 1260 psi. Approximately 7 ml of supernatant was collected by centrifugation at 5000 rev/min 4°C for 15 minutes, and then sterilized with 0.20 μm 26 mm syringe filter (Corning®, NY USA). The sample was loaded onto a 1 ml HPLC Ni<sup>2+</sup> column (GE healthcare, NJ, USA), which was previously charged with 0.1 M NiCl<sub>2</sub> in binding buffer A. A total of 26 fractions were collected at a flow rate of 2.5 ml/min, each fraction containing 500 μl of eluant, of linear gradient with 0% to 100% buffer B at 0.5 ml/min for 10 ml and isocratic flow with 0% buffer A, 100% elution buffer B (50 mM Na<sub>2</sub>HPO<sub>4</sub>, 300 mM NaCl, 500 mM imidazole, pH 8.0) at 0.5 ml/min for 3 ml (protocol of Li: Nad purification method 2.2-NadV Ni purification 2_28_08, Duo Flow HPLC, Grunden Lab, Microbiology dept, NCSU). NadV-His6 protein was eluted at imidazole concentration between 325 and 475 mM. These fractions were analyzed by 12.5% SDS-PAGE. Fractions 14, 15 and 20; 16, 17, 18 and 19; 21, 22 and 23 were combined and dialyzed overnight at 4 °C in 3 L of dialysis buffer (1 mM Tris-HCl, pH 8.0, 1 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM DTT and 10% glycerol), using three Slide-A-Lyzer 3.5 kDa MWCO dialysis cassettes (PIERCE, Rockford, IL, USA). The sample concentration was determined by the Bradford Protein Assay (BIO-RAD, CA, USA). ~ 1 ml recovered for each sample was stored at -80°C with 20% glycerol.
3.2.1.3 NadV and NatV Enzyme-coupled Assay

Enzymatic activities of NadV-His6 and NatV-His6 proteins were measured by a coupled enzyme assay using fluorimetry (5) (21). To detect NAD\(^+\) produced from nicotinamide, fluorescence intensity of NADH was measured using a 340 nm filter for excitation and a 460 nm filter for emission. The coupled enzyme assay conditions used a positioning delay of 0.2 second, 20 flashes per cycle, gain of 80, and cycle time of 20 seconds in fluorescence intensity mode (Polarstar Galaxy, BMG LABTECH, Offenburg, Germany). Total reaction volume was 200 µl in a 96-well Corning microtiter plate with an empty well between each reaction to prevent fluorescence interference. The reaction buffer contained. All the reactions were initiated by adding the enzyme under study.

Since levels of NADH were measured, the alcohol dehydrogenase assay (NAD\(^+\) → NADH) was first standardized. 5 units of alcohol dehydrogenase (ADH; Worthington, NJ, USA) and 1.5% ethanol, along with NAD\(^+\) as a substrate (at final conc. 2.5 mM), were used in a reaction buffer containing 50 mM Tri-HCl, pH 7.64, 12 mM MgCl\(_2\), 0.02 % Bovine Serum Albumin (10) (19) (20). Alcohol dehydrogenase was prepared in 0.1 M phosphate buffer, pH 7.5, filter sterilized with 0.2 µm filter. The ADH assay was performed at 25, 30, and 37°C.

For the NatV assay, nicotinamide mononucleotide adenylyltransferase assay (NMN → NAD\(^+\)), NMN and rATP substrates were added at a final conc. of 2.5 mM each in a reaction with 50 pmole of purified NatV-His6, 5 units of ADH and 1.5% EtOH.

For the NadV nicotinamide phosphoribosyltransferase (NAm → NMN) assay, nicotinamide phosphoribosyl pyrophosphate (PRPP) and nicotinamide were added at 2.5 mM
each and 50 pmole of purified NadV-His6 (19) (20) (24). 50 pmole of NatV-his6, 5 units ADH, and 1.5% EtOH were also supplied in the buffer described above. Note that NMN is omitted from the NadV-NatV-ADH assay.

The reactions were carried out at various temperatures and enzyme amounts to identify optimal conditions. The summary of each assay condition is presented in Table 4.

Table 4. Reaction conditions used in coupled-enzyme assays. The buffer contained 50 mM Tri-HCl, pH 7.64, 12 mM MgCl₂ and 0.02% Bovine Serum Albumin.

<table>
<thead>
<tr>
<th>Component</th>
<th>ADH reaction</th>
<th>NatV reaction</th>
<th>NadV reaction</th>
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<tbody>
<tr>
<td>ADH</td>
<td>5 U</td>
<td>5 U</td>
<td>5 U</td>
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<tr>
<td>EtOH</td>
<td>1.5%</td>
<td>1.5%</td>
<td>1.5%</td>
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<tr>
<td>Substrate</td>
<td>NAD⁺ (2.5 mM)</td>
<td>NMN and ATP (2.5 mM, each)</td>
<td>Nam, PRPP and ATP (2.5 mM, each)</td>
</tr>
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<tr>
<td>NadV</td>
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3.2.1.4 Mass Spectrometry of NatV Reaction

NatV (NMN → NAD⁺) reactions were individually tested by mass spectrometry at the Genomic Science Laboratory (GSL, Centennial Campus, NCSU). All reaction conditions were the same as for the coupled fluorometric assays. Reactions (50 μl) were initiated by adding the enzyme and terminated by adding an equal volume of chilled 100 % MeOH and instantly transferred to an ethanol/dry ice bath. In the NatV reaction, 50 μl mixtures contained buffer (50 mM Tris-HCl, pH 7.5, 12 mM MgCl₂, 0.02 % BSA), 2.5 mM NMN, 2.5
mM rATP and 0.5 µg (12.5 pmole) of NatV-His6 enzyme. The samples were incubated for 0.5 or 1 minute at 25°C. A standard curve of NAD⁺ (0.001 – 0.5 mM) was generated to determine product yield.

3.2.1.5 In vivo NatV Activity

3.2.1.5.1 Preparation of Cell Extracts

NatV (NMNATase) enzyme activity in a proposed pyridine nucleotide (NAD⁺) scavenging pathway was confirmed in KVP40-infected *V. parahaemolyticus* by measuring levels of NADH fluorimetry. EM343 cultures (OD₆₀₀ = ~0.5) were infected with KVP40 at an moi = 7 in a final volume of 12 ml of YP media. One ml of each sample was collected in a 1.5 ml microcentrifuge tube (chilled on ice) before infection then through a time course of 5, 10, 15, 20, 30 and 40 minutes after infection. Pellets from 1 ml infection samples were collected by centrifugation at 10,000 rpm at 4°C, followed by washing with cold TE buffer then stored at -20°C until used. Pellets were resuspended in 100 µl of lysis buffer (50 mM Tris-HCl, pH 7.52, EDTA 1 mM, DTT 1 mM) and disrupted by sonication (20 seconds, three times) on ice. The supernatant was obtained by centrifugation at 10,000 rpm for 5 minutes at 4°C.

3.2.1.5.2 NatV Cellular Activity

Enzymatic activity of NatV protein was measured by a coupled enzyme assay using fluorimetry (21) (5). To detect NAD⁺ produced from nicotinamide, fluorescence intensity of
NADH was measured using a 340 nm filter for excitation and a 460 nm filter for emission. During the assay, provided conditions were established as positioning delay of 0.2 second, 20 flashes per cycle, gain of 80 and cycle time of 20 seconds in fluorescence intensity mode (Polarstar Galaxy, BMG LABTECH, Offenburg, Germany). Total reaction volume was 200 μl in a 96-well Corning microtiter plate with an empty well between each reaction to prevent fluorescence interference. The reaction buffer contained 50 mM Tri-HCl, pH 7.64, 12 mM MgCl₂, and 0.02% Bovine Serum Albumin. The reactions for in vivo enzyme activity were initiated by adding the cell extract under study. The assay was performed at 25°C.

The reaction mixture contained the buffer, 2.5 mM NMN, 2.5 mM ATP, 5 U ADH, 1.5% EtOH and 20 μl of cell extract. For NadV activity, the reaction mixture contained the buffer, 2.5 mM nicotinamide, 2.5 mM PRPP, 2.5 mM ATP, 5 U ADH, 1.5% EtOH and 20 μl of cell extract. The concentrations of total protein of each set at each time point were determined by the Bradford Assay (see Table 7 in section 3.3.1.3).

3.2.1.6 NatV Nudix Hydrolase Assay

The NatV Nudix hydrolase activity was measured by two approaches; indirect and direct. In the indirect method, alkaline phosphatase was used to cleave phosphate from AMP, a product from several Nudix hydrolase substrates, and the released phosphate was measured. In the direct method, Nudix hydrolase specificity was determined by mass spectrometry to measure the yield of AMP.
3.2.1.6.1 Indirect Method: Measuring Phosphate from Nudix Products

A standard curve of phosphate was generated with Na$_2$HPO$_4$ at concentrations of 0.01 to 1 mM. Phosphate solutions (300 μl) of various concentrations were mixed with 700 μl of Pi reagent (1 part 10% ascorbic acid and 6 parts of 0.42% ammonium molybdate·4H$_2$O in 1 N H$_2$SO$_4$), followed by 15 minutes of incubation at 37°C for color development (1). Absorbance at A$_{600}$ was determined. A standard Nudix hydrolase – alkaline phosphatase assay contained 50 mM of Tri-HCl, pH 8.0, 5 mM MgCl$_2$, 1.5 mM of substrate, 5 units of bovine alkaline phosphatase (SIGMA, MO, USA), and 2 μg (50 pmole) of purified NatV-His6. Substrates (all from Sigma-Aldrich, MO, USA) were ADP-ribose (adenosine diphosphate ribose), rATP (adenosine triphosphate), dATP (deoxyadenosine triphosphate), rGTP (guanosine triphosphate), dGTP (deoxyguanosine triphosphate), NAD$^+$ (nicotinamide adenine dinucleotide), NADH (nicotinamide adenine dinucleotide, reduced), NADP$^+$ (nicotinamide adenosine dinucleotide phosphate), NADPH (nicotinamide adenine dinucleotide phosphate, reduced), FAD (flavin adenine dinucleotide) and AP$_3$A (adenosine (5')-triphospho(5')-adenosine, diadenosine triphosphate), all at concentration of 1.5 mM (9) (4). Each microcentrifuge tube contained 300 μl of reaction mixture, incubated at 37°C for 1 minute, then quenched with 700 μl of Pi reagent, followed by 15 minutes incubation at 37°C for color development. When (deoxy) nucleoside triphosphates were used as substrates, the reaction was terminated with 30 μl EDTA (10 mM at final conc.) and 50 μl of 20% activated charcoal (SIGMA) which absorbs residual nucleoside triphosphates, followed by 2 minutes of gentle shaking (23) (16). The supernatant was collected in a new 1.5 ml microcentrifuge tube and then 700 μl of Pi reagent was added. The samples were incubated for 15 minutes at
37°C for color development. The levels of phosphate were then measured at $A_{660}$ with a spectrophotometer (SmartSpec™ 3000, BIO-RAD, CA, USA) (18) (26) (17). Average values were obtained from duplicate samples.

3.2.1.6.2 Direct Method: Mass Spectrometry of Nudix AMP Product

Fifty μl reaction mixtures were prepared that included buffer (50 mM Tris-HCl, pH 8.0, 5 mM MgCl$_2$), 1.5 mM substrate, and 1.08 μg (27 pmole) of NatV-His6 (0.558 μg/μl). The reaction was initiated by adding enzyme. Mixtures were incubated at 37°C for 1 minute and then terminated by adding 50 μl of cold 100% methanol (50% final concentration), followed by rapid transfer to an ethanol/dry ice bath. Samples were stored on dry ice in a -80°C freezer until analyzed by mass spectrometry at the NCSU GSL. An injection volume of 5 μl was used. A standard curve of AMP (0.5 μM - 0.5 mM conc. in 50% MeOH) was generated.

3.2.2 Expression Analysis of mRNA during KVP40 Infection

3.2.2.1 Strains and Phage Infection

KVP40 and its host, *Vibrio parahaemolyticus* (EM343), were isolated by Matsuzaki et al. and obtained from Hans Ackermann of the University of Laval (Quebec, Canada) (12). KVP40 lysates were prepared as follows: Overnight grown EM343 was diluted to a starting
OD$_{600}$=0.1. The culture was grown 2–3 hours until the OD$_{600}$ reached ~0.5. Meanwhile, YP plates (0.3% of Yeast extract, 1% of Peptone, 3% NaCl, 2% agar) were warmed to room temperature. 100 μl of EM343 and a single plaque from a plate were combined in 3 ml of molten, 48°C YP medium with agar (0.4%). YP top agar containing EM343 and KVP40 was poured and distributed on a pre-warmed YP plate and allowed to solidify. The plate was incubated at 32°C overnight. The next day, the top agar was scraped from the plate and the plate rinsed with 1 ml of YP broth medium to collect the residue. The collected agar was combined in a 50 ml conical centrifuge tube. 500 μl of chloroform was added to the tube, mixed and incubated at room temperature for 5 minutes to enhance lysis. The supernatant was collected by centrifugation (Sorvall® RC-5B Refrigerated Superspeed Centrifuge, DuPont) at 5,000 rev / min for 10 minutes at 4°C. The supernatant lysate of KVP40 was removed and stored at 4°C with a few drops of chloroform before titering. The lysate pfu/ml titer was determined by standard plate plaque assays using mid-log EM343 cells and YP media.

For mRNA analysis during infection, EM343 cultures (OD$_{600}$ = ~0.5) were infected with KVP40 at an moi = 7 in a final volume of 12 ml of YP media. One ml of each sample was collected in a 2 ml microcentrifuge tube containing 0.4 ml of RNAlater (see Appendix B for recipe). Samples were collected before infection, then through a time course of 1, 3, 5, 8, 10, 15, 20, 30 and 40 minutes after infection. The samples were incubated with RNAlater for at least 5 minutes on ice during collection. Samples were centrifuged at 5,000 g and washed with 0.4 ml of TE buffer. Pellets were immediately used to extract RNA.
3.2.2.2 RNA Isolation and DNase Treatment

Freshly prepared 1 ml samples of infected cell pellets, resuspended in 1 ml TE buffer containing 0.4 mg lysozyme, were used for total RNA extraction with an RNeasy Mini Kit (QIAGEN, CA, USA). RNA was eluted with 30 µl of nuclease-free water. Samples were then treated with 8 units of DNase RQ1 (Promega, WI, USA) and incubated at 37°C for 20 minutes in a total volume of 80 µl, followed by 10 minutes of enzyme inactivation at 65°C. The elimination of DNA was confirmed by running samples on 1% agarose-TBE gels and by PCR using phage-specific primers for KVP40 genes 23 and frd. 200 ng of total RNA was used in confirmation of DNA background removal. Concentration of total RNA (A260) was determined with a NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, MA, USA) in triplicate readings of 2 µl samples. Two µl of 1:6 diluted RNA samples in DEPC-ddH2O was loaded on the lens of the NanoDrop and the average was obtained from triplicate readings.

3.2.2.3 cDNA Synthesis and Reverse Transcription

500 ng of total RNA was reverse transcribed using 100 units of M-MLV reverse transcriptase (Promega, WI, USA), 0.25 µg of random hexamer (New England Biolabs, MA, USA) and 20 units of RNaSin (Promega, WI, USA) in a final volume of 25 µl. The reaction was incubated at 37°C for 1 hour, followed by 10 minutes enzyme inactivation at 80°C.
3.2.2.4 Quantitative Real Time-PCR

0.4 ng equivalent of cDNA, based on the initial 500 μl of RNA added to the reverse transcription reactions, was used in qRT-PCR reactions. Nine different phage-specific primer sets and *V. parahaemolyticus* 16S rRNA primers as an internal control, were used. The primer sets were designed to amplify 100-150 bp products using software at idtdna.com; the sequences are displayed in Table 5. The reaction volume was established as 10 μl. Real-time PCR was performed in an iCycler (BIO-RAD, CA, USA) using SYBR Green (Absolute Blue™, Thermo scientific, MA, USA). The reaction was performed in triplicate samples with each gene primer set. Forward and reverse primers were added at a final concentration of 2.5 μM. The amplification conditions included 15 minutes of enzyme activation at 95°C, 40 cycles of denaturing at 95°C for 30s, 51°C for 30s, and 72°C for 1 min, followed by a melt curve of 95°C for 1 min, 55°C for 1 min and increasing the set point temperature after cycle 2 by 0.5°C every 10s. *nadV*, 23, *frd* and 55 primers were used to amplify KVP40 genomic DNA as a calibrator in the calculation of mRNA copies (See Appendix C).
Table 5. KVP40 and Vibrio parahaemolyticus EM343 primer sequences used in qRT-PCR. Melting temperature and GC content of each primer are presented.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequences</th>
<th>Product size (bp)</th>
<th>Tm</th>
<th>GC %</th>
</tr>
</thead>
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<td>natVqRT5'</td>
<td>5'-TAGCTCAGTGCGCGAAATCG-3'</td>
<td>106</td>
<td>58.8</td>
<td>55</td>
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<tr>
<td>natVqRT3'</td>
<td>5'-GAGAAGGACATGACCTGCAC-3'</td>
<td>56.3</td>
<td>55</td>
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<tr>
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<td>55</td>
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<td>50</td>
<td></td>
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<tr>
<td>23qRT5'</td>
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<td>124</td>
<td>57.3</td>
<td>55</td>
</tr>
<tr>
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<td>55</td>
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<tr>
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<td>58.9</td>
<td>66.7</td>
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</tbody>
</table>

3.2.2.5 qRT-PCR Analysis

Using the 2ΔΔ^C_T method of Clokie et al., the absolute copy number of mRNA at each infection cycle time point was calculated (see Appendix C for detail) (3). C_T values were acquired at a threshold set at 1/3 of the max RFU (relative fluorescent units). An average C_T value was obtained from triplicate assays. Outlier data were detected and excluded using the Grubbs’ test (http://www.graphpad.com).
3.3. Results

3.3.1 NadV and NatV Enzyme Characteristics

3.3.1.1 NadV and NatV Enzyme Purification

3.3.1.1.1 His6-tagged NatV Overexpression and Purification

NatV expression plasmids pZL166/HK80 and pZL176/HK90 (Z. Li, MS Thesis NCSU) were transformed into BL21-AI (Invitrogen, CA, USA) cells (Figure 11). Expression was induced by addition of 1 mM IPTG and 0.2% L-arabinose with extra 4 hour incubation. Although Li was not able to show differences between induced sample and un-induced sample, in this experiment, the induced sample displayed a significantly thicker band than un-induced sample on 12.5% SDS gel (data not shown). Based on its amino acid sequence, the expected size of expressed NatV-His6 is ~ 40.2 kDa. One 8 ml BL21-AI/pZL166 sample and four 8 ml BL21-AI/pZL176 samples, thus total 40 ml combined pellets resuspended in 8 ml of binding buffer were lysed and His6-tagged NatV was successfully purified using a Ni\(^{2+}\)-charged affinity column (GE healthcare, NJ, USA) and elution buffer. Forty five fractions (0.5 ml each) were collected. The results indicated one major UV-absorbing peak (fractions 10 ~ 13) and one small bump (fractions 26 ~31). Fraction numbers 27, 28, 29, 30 and 31 showed strong bands at ~ 40 kDa, while fractions 12 ~ 14 showed numerous background bands on 12.5% SDS-PAGE. Fraction number 24, 25 and 26 (group 1); 27, 28, 29, 30 and 31 (group 2); and 32, 33, 34 and 35 (group 3) were combined and the three groups were dialyzed overnight. 1.8 ~ 2.7 ml volumes were recovered for each group. The concentration of each fraction group was determined by the Bradford assay (Table 6) and
analyzed by SDS-PAGE (Figure 12).

Figure 11. natV expression plasmid pZL166 and pZL176 (11).

Table 6. Concentration of purified His6-tagged NatV and His6-tagged NadV determined by the Bradford assay (µg/µl).

<table>
<thead>
<tr>
<th></th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>NatV-His6</td>
<td>0.125</td>
<td>0.558</td>
<td>0.118</td>
</tr>
<tr>
<td>NadV-His6</td>
<td>0.0985</td>
<td>0.113</td>
<td>0.105</td>
</tr>
</tbody>
</table>

48
Figure 12. Samples from NatV-His6 purification analyzed by 12.5% SDS-PAGE. Lane 1, Broad range protein marker (Bi-Rad, CA, USA); lane 2, induced BL21-AI/pZL166 whole cells; lane 3, induced cell extract; lane 4, fraction number 12 from HPLC; lane 5, fractions 24, 25 & 26 (group 1); lane 6, fractions 27, 28, 29, 30 & 31 (group 2; used in the enzyme assay); lane 7, fractions 32, 33, 34 & 35 (group 3).

3.3.1.1.2 His6-tagged NadV Expression and Purification

KVP40 nadV was previously cloned into pET101/D-TOPO downstream of the T7 promoter and transformed into TOP10 cells to produce pZL405 (Figure 13, Z. Li, M.S. Thesis NCSU). NadV-His6 expression was induced as described in Materials and Methods.
Figure 13. *nadV* expression plasmid pZL405. It was constructed utilizing the pET101/D-TOPO vector with *nadV* gene insertion (1516 bp) as shown (Z. Li., M.S. Thesis NCSU) (11).

Five ml induced cell pellets were resuspended in 8 ml of binding buffer A, lysed by French Press and NadV-His6 successfully purified on a Ni$^{2+}$-charged affinity column. Fractions collected (see section 3.2.1.2) were analyzed on 12.5% SDS-PAGE to verify the presence of NadV-His6 protein (expected size of 55.55 kDa, Figure 14). Although the HPLC elution results displayed a significant peak at fractions from 6-8, the NadV-His6 protein was actually present at later fractions 14 to 23. Fractions 14, 15 and 20 (group 1); 16, 17, 18 and
19 (group 2); and 21, 22 and 23 (group 3) were combined and then dialyzed overnight; 1 ~ 2 ml samples were recovered. The concentration of each fraction group was determined by the Bradford assay (Table 6).

**Figure 14.** Samples from NadV-His6 purification analyzed by 12.5% SDS-PAGE. Lane 1, Bio-Rad Broad range protein marker; lane 2, induced BL21-Al/pZL405 TOP10 cells; lane 3, induced cell extract; lane 4, fraction 7 from HPLC; lane 5, fractions 14, 15 & 20 (group 1); lane 6, fractions 16, 17, 18 & 19 (group 2; used in the enzyme assay); lane 7, fractions 21, 22 & 23 (group 3).
3.3.1.2 NadV and NatV Coupled-enzyme Assay

In KVP40, the proposed two-reaction NAD\textsuperscript{+} scavenging pathway requires two enzymes, NadV (Nicotinamide phosphoribosyltransferase) which converts nicotinamide to nicotinamide mononucleotide in the presence of phosphoribosyl pyrophosphate (PRPP), and NatV (NMN adenylyltransferase, C-terminal domain) which catalyzes conversion of NMN and ATP to NAD\textsuperscript{+}. The two purified His6-tagged enzymes were assayed by two methods; reactions coupled to alcohol dehydrogenase (ADH), and by direct mass spectrometry of reaction products. Coupled enzyme assays were performed by the method of Revollo et al. 2004 (19). Fluorescence of NADH was measured, as an indicator of NAD\textsuperscript{+} production, by excitation at 340 nm and emission at 460 nm, where NAD\textsuperscript{+} produced by NatV is converted to NADH by alcohol dehydrogenase. In a three-step reaction, NADH was synthesized from initial substrates, nicotinamide and PRPP. However, each stage of the reaction was standardized and verified.

3.3.1.2.1 Standardizing the Alcohol Dehydrogenase Reaction: NAD\textsuperscript{+} → NADH

A linear response of NADH fluorescence was continued in a standard curve of NADH between 0 and 100 μM (Figure 15). Since levels of NAD\textsuperscript{+} cannot be measured directly, reduced form of NAD\textsuperscript{+}, NADH, was measured. 5 units of alcohol dehydrogenase (ADH) and ethanol at 1.5% final concentration were used in the reaction. The standard ADH reaction was started by adding 5 units of ADH to buffer, containing 2.5 mM NAD\textsuperscript{+} and ethanol at a final concentration of 1.5%. The rapid reaction reached maximum within a couple minutes. By using various amounts of ADH - 0.5, 1, 2.1 and 5 units - the affect of
ADH on the production of NADH from 2.5 mM of NAD⁺ at 25°C was demonstrated (Figure 16). NADH production in for this particular experiment with various amount of ADH presented 94 μmole/sec and 220 μmole/sec, when 0.5 U and 1 U ADH were added, respectively. Temperatures of 25, 30 and 37°C did not substantially differentiate levels of NADH produced (Figure 17). NADH production per second was 52 μmole, 42 μmole and 46 μmole, at 25°C, 30°C and 37°C, respectively, when 1U ADH was used.

\[ y = 17.229x + 49.951 \]

Figure 15. Fluorescence of NADH standard solutions. Only NADH concentrations between 0 and 100 μM give a linear response. The average was obtained from duplicate samples.
Figure 16. NADH production from NAD$^+$ with various amounts of ADH. The reaction mixture included 2.5 mM NAD$^+$ and 1.5% ethanol. The assay was performed at 25°C. In this assay, the ADH rate was 94 μmole NADH/sec/U ADH or 220 μmole NADH/sec/U ADH at 25°C, when 0.5 U or 1 U ADH were respectively used in the reaction. ADH specific activity with 2 U and 5 U of ADH was out of range in the NADH standard curve. The specific activity was obtained within the first 20 seconds. The data were obtained from a single experiment.
Figure 17. ADH reactions at 25, 30 and 37°C. The reaction mixture contained 2.5 mM of NAD\(^+\), 1.5% ethanol and 5 units of alcohol dehydrogenase. In this assay, the ADH rate was 52 μmole NADH/sec/U ADH at 25°C, 42 μmole NADH/sec/U ADH at 30°C and 46 μmole NADH/sec/U ADH at 37°C. ADH specific activity was obtained within the first 20 seconds during the reaction. The average was obtained from duplicate assays.

3.3.1.2.2 NatV NMN Adenylyltransferase Reaction: NMN → NAD\(^+\)

The NatV NMNATase reaction mixture contained 2.5 mM NMN and ATP, 50 pmole NatV-His6 and 5 units of ADH and 1.5% ethanol at 25°C. The reaction was commenced by adding NatV enzyme to the mixture. NADH was produced from NMN by NadV NMNATase activity, as shown by the increasing fluorescence level (Figure 18). The specific activity of NatV enzyme (50 pmole) was calculated within the first 20 seconds of the reaction. The specific NMNATase activity at 25°C was 50 μmole/sec/μg NatV-His6.
Figure 18. Purified NatV-His6 NMNATase activity. NADH synthesized by NatV from NMN at 25°C. The reaction mixture contained 2.5 mM NMN, 2.5 mM ATP, 50 pmole NatV, 1.5% ethanol and 5 units of alcohol dehydrogenase. The reaction rate for NatV was 50 μmole of NADH/sec/1 μg of NatV-His6 at 25°C. NatV specific activity was obtained within the first 20 seconds when 50 pmole of NatV was used. The data are average of duplicate assays. The specific activity was 50 μmole NADH+/sec/μg NatV-His6

3.3.1.2.3 Confirmation of NatV NMNATase Activity by Mass Spectrometry

The NatV NMNATase activity was authenticated by mass spectrometry (GSL, NCSU). The reaction condition was maintained the same as in the coupled enzyme assay (see section 3.3.1.2.2), except that NADH+ was detected directly. With 12.5 pmole NatV provided, NMN was converted to NADH+ in the presence of ATP, yielding 5.2 mmole and 6.3 mmole of NADH+ in 0.5 and 1 minute, respectively at 25°C. The average was obtained from duplicate samples. The maximum specific activity (0.5 minutes, 25°C) was 350 μmole/sec/μg NatV-
3.3.1.2.4 NadV Nicotinamide Phosphoribosyltransferase Reaction: NAm → NMN

A three-step “coupled” assay system with NadV, NatV and ADH was performed. In this reaction, NadV would convert nicotinamide and PRPP to NMN, NMN plus ATP would be converted to NAD$^+$ by NatV and then ADH would yield the fluorescent NADH. The reaction mixture contained 2.5 mM PRPP, 50 pmole NatV, 2.5 mM rATP, 5 units of ADH and ethanol (1.5%). The reaction was initiated by adding 50 pmole of NadV at 25°C. In this reaction, the rate was slow compared to the NatV-ADH reaction.

The assay was performed with various amounts of NadV at 10, 25, 50, 75 and 100 pmole (Figure 19). When 10, 25 or 40 pmole of NadV was used, NADH production was significantly reduced within a given assay period. With 50 or 100 pmole of NadV, NADH yield was maximum at approximately 3 or 4.5 mmole after 13 minutes of incubation, respectively. However, in the NadV-NatV coupled enzyme reaction, NADH production was delayed 2 ~ 6 minutes. The coupled-reaction with 10, 25 or 40 pmole NadV did not show NADH production until 6 minutes after the reaction was initiated. The reaction containing 50 pmole of NadV showed the earliest NADH production, although the NadV specific activity was the greatest with 100 pmole of NadV. The NadV specific activity showed 0.08, 0.13, 0.17, 0.32, and 0.51 mmole of NADH produced per minute by 10, 25, 40, 50 and 100 pmole of NadV, respectively at 25°C. The enzyme specific activity was obtained within 13 minutes of assay period and with the specific amount of NadV supplied. Reaction temperature did not
influence the levels of NADH produced. The coupled-enzyme assay performed at 25, 30 and 37°C showed no significant differences (Figure 20). NADH produced per second by 1 μg of NadV was 5.2, 4.9 and 5.0 μmole at 25°C, 30°C and 37°C, respectively.

![Figure 19](image-url)

**Figure 19.** Purified NadV-His6 NAmPRTase activity. NADH production from nicotinamide in a three-component coupled enzyme assay. The reaction mixture contained various amounts of NadV, 2.5 mM PRPP, 50 pmole NatV, 2.5 mM rATP, 5 units of ADH and 1.5% ethanol, at 25°C. NMN produced per minute was 0.08 mmole (10 pmole NadV), 0.13 mmole (25 pmole NadV), 0.17 mmole (40 pmole NadV), 0.32 mmole (50 pmole NadV) or 0.51 mmole (100 pmole NadV) at 25°C. NadV specific activity was obtained within 13 minutes of assay period. NMN produced (μmole)/sec/μg NadV-His6 was 2.7 (10 pmole NadV), 1.5 (25 pmole NadV), 1.3 (40 pmole NadV), 1.8 (50 pmole NadV) or 1.5 (100 pmole NadV). Data are from a single experiment (duplicate for 40 and 50 pmole NadV).
Figure 20. Purified NadV-His6 NAmPRTase activity at various temperatures. The reaction mixtures contained 50 pmole NadV, 2.5 mM PRPP, 50 pmole NatV, 2.5 mM ATP, 5 units of ADH and 1.5% ethanol. The specific activities were 5.2 (25°C), 4.9 (30°C) and 5 (37°C) μmole NMN/sec/μg NadV-His6. NadV specific activity was obtained within the first 100 seconds. The average was obtained from duplicate assays.

3.3.1.3 In vivo NatV Enzyme Activity

NatV NMNATase activity was confirmed in KVP40-infected *V. parahaemolyticus* cells. Levels of NADH were measured by fluorometry using a 340 nm excitation filter and 460 nm emission filter. The concentration of total protein at each time point was presented in Table 7. The data were obtained from duplicate samples per each set, two sets total. Levels of NADH during infection, presented in Figure 21, showed that the KVP40-infected *V. parahaemolyticus* has NatV NMNATase activity that converts nicotinamide mononucleotide
and ATP to NAD$^+$. By providing ADH in the assay, the NAD$^+$ can be converted to NADH. The NatV specific activity, measured by NADH produced per second with 1 μg of cell extract, was maximum at 15-20 minutes after infection.

**Table 7.** Protein concentrations of KVP40-infected *V. parahaemolyticus* cell extracts determined by the Bradford assay (μg/μl). BI, before infection; T1-T40, minutes after infection.

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</tbody>
</table>

**Figure 21.** NMNATase activity in KVP40-infected cells. NADH (μmole) produced per second with 1 μg of total protein. The samples were from a single infection and the average was obtained from duplicate assays per each set.
3.3.1.4 Characterization of the NatV Nudix Hydrolase Activity

The C-terminal half of NatV is predicted to be a Nudix hydrolase domain that is homologous to Synechocystis sp. slr0787, a protein that also possesses bifunctional NMNATase/Nudix hydrolase activity. Nudix hydrolase activity of NatV was measured by two methods. In one approach, alkaline phosphatase is used to release a terminal phosphate from one of the Nudix hydrolase products, adenosine monophosphate (AMP). Levels of inorganic phosphate are then measured by a spectrocolorimetric assay. Nudix hydrolase products were also directly assayed using mass spectrometry.

3.3.1.4.1 Phosphate Assay: Indirect Nudix Hydrolase Assay

NatV Nudix hydrolase specificity was evaluated on a range of substrates, including ADP-ribose, NAD⁺, NADH, NADP⁺, NADPH, rATP, dATP, rGTP, dGTP, FAD and AP₃A. A phosphate standard curve was first generated using Na₂HPO₄ (Figure 22) and the ascorbic acid-ammonium molybdate reagent (1). NatV (1 μg) reaction mixtures contained buffer, 1.5 mM substrate incubated and processed as described in section 3.2.1.6. The evaluated Nudix hydrolase substrates and respective reaction products were:

\[
\begin{align*}
\text{ADP-ribose} + H_2O & \rightarrow \text{AMP} + \text{ribose-P} \\
\text{NAD}^+ + H_2O & \rightarrow \text{AMP} + \text{NMN} \\
\text{NADH} + H_2O & \rightarrow \text{AMP} + \text{NMNH} \\
\text{NADP}^+ + H_2O & \rightarrow \text{AMP} + \text{NMNP} \\
\text{NADPH} + H_2O & \rightarrow \text{AMP} + \text{NMNPH}
\end{align*}
\]
\[
\begin{align*}
\text{rATP} + \text{H}_2\text{O} & \rightarrow \text{AMP} + \text{PPi} \\
\text{dATP} + \text{H}_2\text{O} & \rightarrow \text{dAMP} + \text{PPi} \\
\text{rGTP} + \text{H}_2\text{O} & \rightarrow \text{GMP} + \text{PPi} \\
\text{dGTP} + \text{H}_2\text{O} & \rightarrow \text{dGMP} + \text{PPi} \\
\text{FAD} + \text{H}_2\text{O} & \rightarrow \text{AMP} + \text{FMN} \\
\text{AP}_3\text{A} + \text{H}_2\text{O} & \rightarrow \text{AMP} + \text{ADP}
\end{align*}
\]

In the indirect assay, \(\text{Pi}\) is cleaved from AMP by alkaline phosphatase present in the reaction mixture.

Figure 22. A phosphate standard curve for Nudix hydrolase assays. The ascorbic acid-ammonium molybdate assay was used (Ames and Dubin). The average was obtained from duplicate samples.
When 1 µg of NatV-His6 was provided in the reaction, 0.6, 0.6, 0.5 and 0.3 µmole of AMP was produced per second from ADP-ribose, NADH, NAD\textsuperscript{+} and NADPH, respectively (Figure 23). The amount of AMP produced from (Deoxy) nucleoside triphosphates; rATP, dATP, rGTP and dGTP, was not significant. AMP production from FAD and AP\textsubscript{3}A was also fairly low. Overall, ADP-ribose, NADH and NAD\textsuperscript{+} were indicated to be preferred substrates of NatV Nudix hydrolase.

**Figure 23.** Substrate specificity of KVP40 Nat V Nudix hydrolase measured by the phosphate release assay. 1.5 mM of substrate and 50 pmole NatV were present in each sample. Specific NatV activity was determined by Abs at 660 nm after subtraction of background. The amount of AMP was calculated based on Pi produced/min by 25 pmole of NatV Nudix hydrolase at 37°C. In this assay, the rate was 0.6 µmole AMP/sec/µg NatV-His6 for ADP-ribose, 0.5 µmole AMP/sec/µg NatV-His6 for NAD, and 0.6 µmole AMP/sec/µg NatV-His6 for NADH at 37°C. The average was obtained from duplicate assays. Stoichiometry of AMP (dAMP for dATP, GMP for GTP and dGMP for dGTP) to phosphate is 1:1.
3.3.1.4.2 Mass Spectrometry: Direct Nudix Hydrolase Assay

For Nudix hydrolase product analysis (AMP) by mass spectrometry, preferred substrates in the phosphate release assay were incubated (1min, 37°C) with 25 pmole NatV-His6. Results from mass spectrometry are presented in Figure 24. ADP-ribose showed the highest AMP production, followed by FAD, NADH and NAD+. AMP production from the remaining substrates, including NADP, NADPH, rATP and dATP was not detected. Note that some substrates (i.e., NADPH, NADP+, AP₃A) showing reactivity in the indirect assay did not show AMP release by mass spectrometry. The specific activities on the preferred substrates were calculated from the mass spectrometry and measurements of AMP production were: ADP-ribose - 0.6 μmole/sec/μg NatV-His6; FAD - 0.2 μmole/sec/μg NatV-His6; NADH - 0.19 μmole/sec/μg NatV-His6; and NAD⁺ - 0.15 μmole/sec/μg NatV-His6.
Figure 24. Mass spectrometry analysis of KVP40 NatV Nudix hydrolase substrate specificity. The enzyme specific activity was obtained with 1.5 mM substrate and 1 μg NatV, incubated at 37°C. The average was obtained from duplicate assays (triplicate for ADP-ribose). The rate of NatV on ADP-ribose was 0.6 μmole AMP/sec/μg NatV-His at 37°C.

3.3.2 Expression Patterns of *nadV* and *natV* during KVP40 Infection

Quantitative real-time PCR was used to evaluate *nadV* and *natV* transcription levels during infection. In total, nine KVP40 genes and *Vibrio parahaemolyticus* 16S rRNA genes as an internal control were analyzed. Transcription analysis for this phage has not been previously conducted. Based on established characterizations of the expression of T4 homolog genes, potential early and late period genes were chosen. These were: *td* (early), *asiA* (early), *frd* (early), 55 (late), 25 (late), *wac* (late) and 23 (late). Expression of *nadV*,
natV, td, asiA and frd were expected to occur early based on their roles in cell metabolism, whereas 25, wac and 23 are structural components of the KVP40 virion and were expected to be transcribed late. Gene 55 encodes a phage sigma factor for late transcription and was expected to appear during the early-late transcription period. The functions of these genes are listed in Table 8.

Table 8. KVP40 genes used in qRT-PCR analysis. Genes were chosen based on their functions and relative expression time in T4 (15).

<table>
<thead>
<tr>
<th>KVP40</th>
<th>Description</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>nadV</td>
<td>Nicotinamide phosphoribosyltransferase</td>
<td>Proposed pyridine scavenging</td>
</tr>
<tr>
<td>natV</td>
<td>NMN adenylyltransferase</td>
<td>Proposed pyridine scavenging</td>
</tr>
<tr>
<td>td</td>
<td>Thymidylate synthase</td>
<td>Nucleotide metabolism</td>
</tr>
<tr>
<td>asiA</td>
<td>Anti-sigma 70</td>
<td>Inhibits transcription</td>
</tr>
<tr>
<td>frd</td>
<td>Dihydrofolate reductase</td>
<td>Nucleotide metabolism</td>
</tr>
<tr>
<td>55</td>
<td>Sigma factor</td>
<td>Late transcription</td>
</tr>
<tr>
<td>25</td>
<td>Baseplate wedge</td>
<td>Tail, tail fiber</td>
</tr>
<tr>
<td>wac</td>
<td>Whiskers</td>
<td>Tail, tail fiber</td>
</tr>
<tr>
<td>23</td>
<td>Major capsid protein</td>
<td>Head</td>
</tr>
</tbody>
</table>
3.3.2.1 RNA Isolation and Genomic DNA Removal

KVP40 was used to infect *Vibrio parahaemolyticus* strain EB101 (EM343) at an moi (multiplicity of infection) of 7. One ml sample for each time point before and after infection was collected into RNAlater and RNA was subsequently extracted. Isolated RNA samples were treated with DNase to remove genomic DNA, and then the quality of RNA was determined by gel electrophoresis and NanoDrop® ND-1000 UV-Vis Spectrophotometer (Table 9). The presence of genomic DNA can interfere with synthesized cDNA in qRT-PCR analysis. Gel analysis of RNA samples showed two major bands of 16S and 23S ribosomal RNA; little to no large molecular weight nucleic acid genomic DNA was observed (Figure. 25). In repeated preparations, more RNA was present at 3 ~ 15 minutes after infection than 20 ~ 40 minutes after infection. This may indicate the peak expression period or the onset of cell lysis at later times.

### Table 9. Concentrations of DNase-treated RNA determined by NanoDrop UV spectrophotometry. The concentration of total RNA was obtained from the average of triplicate readings with 2 μl samples. BI, before infection; T1-T40, minutes after infection (ng/μl).

<table>
<thead>
<tr>
<th></th>
<th>BI</th>
<th>T1</th>
<th>T3</th>
<th>T5</th>
<th>T8</th>
<th>T10</th>
<th>T15</th>
<th>T20</th>
<th>T30</th>
<th>T40</th>
</tr>
</thead>
<tbody>
<tr>
<td>BI</td>
<td>82.64</td>
<td>155.86</td>
<td>185.42</td>
<td>252.9</td>
<td>226.64</td>
<td>217.52</td>
<td>262.32</td>
<td>142.92</td>
<td>233.22</td>
<td>137.78</td>
</tr>
</tbody>
</table>
Elimination of genomic DNA can be confirmed by qRT-PCR with RNA before cDNA synthesis. 200 ng total RNA was amplified in an iCycler (Bio-Rad, CA, USA) with two phage-specific primer sets, (23 and frd), in parallel with 10 ng of genomic DNA as a positive control. qRT-PCR results showed little to no amplification product with RNA only and strong amplification with genomic DNA. The qRT-PCR products were also analyzed on 1% agarose-TBE gels and confirmed the absence of amplification products using RNA only (Figure 26).
Figure 26. Samples of RNA and KVP40 genomic DNA used in qRT-PCR to confirm the absence of amplification. Lane 1, Promega 100bp DNA ladder; lane 2, T3 with 23; lane 3, T10 with 23; lane 4, T30 with 23; lane 5, T3 with frd; lane 6, T10 with frd; lane 7, T30 with frd; lane 8, KVP40 genomic DNA with 23; lane 9, KVP40 genomic DNA with frd.

3.3.2.2 cDNA Synthesis

500 ng of total RNA were converted to cDNA using M-MLV reverse transcriptase and random hexamer primers. The reaction was incubated at 37°C for 1 hour, followed by enzyme inactivation at 80°C. Since cDNA yield can not be determined directly in these reactions due to interference of dNTP, cDNA concentrations were routinely based on 1:1 conversion of RNA to cDNA. A negative control without RNA was processed.
3.3.2.3 Quantitative Real Time PCR (qRT-PCR) Analysis using the $2\Delta\Delta^CT$ Method

0.4 ng equivalent of cDNA was transferred from the reverse transcription reactions into qRT-PCR with 9 different phage-specific and 16S rRNA primer sets. 16S rRNA was used to normalize the $C_T$ values of the phage gene transcripts. Forward and Reverse primers (2.5 μM each) were combined for a final concentration of 0.4 μM in a 10 μl total reaction volume. All nine single-copy phage genes were first tested on genomic DNA and showed relatively close $C_T$ values. The same amount of purified genomic DNA with each phage gene was run in parallel as a positive control for each set of primers. All qRT-PCRs were done in triplicate and the average of three data sets was presented. Triplicate samples were run on the same plate and each plate included 16S rRNA reactions. The number of gene transcripts for each gene was calculated by the $2\Delta\Delta^CT$ method of Clokie et al. (Appendix C). During infection, the range of raw $C_T$ values for phage transcripts varied from 21.51 to 35.75; however $C_T$ values greater than 30 cycles, where the graph plateaus, are considered to be background (http://www.ncbi.nlm.nih.gov/projects/genome/probe/doc/TechQPCR.shtml). The average 16S rRNA $C_T$ value was 12.78, excluding one outlier (16.02) by the Grubbs’ test. Genomic DNA amplified with $nadV$, 23, $frd$ and 55 primers yielded average $C_T$ values that were used as a calibrator in the calculation of transcript copy. There were no phage transcripts detected before infection or at one minute after infection. Example raw $C_T$ plots of the qRT-PCR data are shown in Appendix D.

Transcripts of $nadV$ first appeared after 3 minutes of infection, reached a peak at 20, then decreased. $natV$ also first appeared after 3 minutes post-infection, peaked at 10 minutes, then gradually diminished. KVP40 $td$ displayed a similar pattern, with maximal expression at
8 minutes then gradually diminishing by 20 minutes after infection and tended to persist. mRNA copies of these three genes are plotted over the time of infection in Figure 27A.

Transcripts of asiA emerged after those mentioned above, exhibiting a maximum at 15 minutes. Both frd and gene 55 mRNA first appeared at 5 to 8 minutes after infection. frd reached near maximum at 15 minutes, peaked at 30 minutes, and dramatically dropped at 40 minutes. Gene 55 displayed a maximum at 20 minutes after infection. From these observations, asiA, frd, and 55 can be considered “delayed early” genes in their expression patterns (Figure 27B).

Gene 23 demonstrated the highest mRNA level (1.43 x 10^6 mRNA copies at 30 minutes) among the nine phage genes and gene 25 showed the least amount of mRNA transcript (2.69 x 10^4 copies). These gene transcripts, including those of wac, appeared 10 to 15 minutes post-infection and increased until 30 to 40 minutes. These are clearly “late genes” with respect to their expression pattern (Figure 27C).
A.

B.

C.
Figure 27. Absolute mRNA levels of KVP40 genes analyzed using qRT-PCR. Genes were grouped according to their expression periods as, early, delayed early and late. A. Expression patterns of KVP40 early genes nadV, natV and td. B. Expression patterns of KVP40 delayed early genes asiA, frd and g55. C. Expression patterns of KVP40 late genes 25, 23 and wac. The average was obtained from triplicate assays.

Relative transcription patterns of each gene were also plotted and compared (Figure 28A, B and C); nadV and natV, parallel with td, were detected 3 to 5 minutes after infection as “early” genes; asiA, frd and g55 are appeared 5 to 8 minutes after infection as “delayed early” genes; and 23, 25 and wac emerged 10 to 15 minutes after infection as “late” genes. As there is no T4-like “middle-mode” MotA activator in KVP40, asiA, frd and g55 were designated as “delayed early” genes. The transcription patterns of the KVP40 genes are summarized in Table 10. The raw C_T values from each primer sets were presented in Appendix D.

Table 10. Summary of qRT-PCR expression data from KVP40-infected V. parahaemolyticus.

<table>
<thead>
<tr>
<th>Transcription pattern</th>
<th>Gene</th>
<th>Appearance</th>
<th>Peak Time</th>
<th>No. of transcripts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early</td>
<td>nadV</td>
<td>T3-5</td>
<td>T20</td>
<td>1.9 x 10^5</td>
</tr>
<tr>
<td></td>
<td>natV</td>
<td>T3-5</td>
<td>T10</td>
<td>6.0 x 10^5</td>
</tr>
<tr>
<td></td>
<td>td</td>
<td>T3-5</td>
<td>T8</td>
<td>2.7 x 10^3</td>
</tr>
<tr>
<td>Delayed early</td>
<td>asiA</td>
<td>T5-8</td>
<td>T15</td>
<td>1.3 x 10^5</td>
</tr>
<tr>
<td></td>
<td>frd</td>
<td>T5-8</td>
<td>T30</td>
<td>3.7 x 10^5</td>
</tr>
<tr>
<td></td>
<td>g55</td>
<td>T5-8</td>
<td>T20</td>
<td>1.8 x 10^5</td>
</tr>
<tr>
<td>Late</td>
<td>g25</td>
<td>T10-15</td>
<td>T20</td>
<td>2.7 x 10^4</td>
</tr>
<tr>
<td></td>
<td>g23</td>
<td>T10-15</td>
<td>T30</td>
<td>1.4 x 10^6</td>
</tr>
<tr>
<td></td>
<td>wac</td>
<td>T10-15</td>
<td>T30</td>
<td>1.2 x 10^5</td>
</tr>
</tbody>
</table>
Figure 28. Relative mRNA levels of KVP40 genes analyzed using qRT-PCR. Genes were grouped according to their expression periods as early, delayed early. Relative expression is percent maximum transcript level for each gene. A. Expression patterns of KVP40 early genes nadV, natV and td. B. Expression patterns of KVP40 delayed early genes asiA, frd and 55. C. Expression patterns of KVP40 late genes 25, 23 and wac. The average was obtained from triplicate assays.
3.4. Discussion

3.4.1 NadV and NatV Enzyme Characterization

3.4.1.1 NadV-His6 and NatV-His6 Expression and Purification

In this work, NatV-His6 was expressed in a manner similar to that used for NadV-His6 (Z. Li, M.S. Thesis, NCSU) by adding IPTG, L-Arabinose, and D (+)-glucose. SDS-PAGE results verified a prominent band of expressed NatV-His6 protein at ~ 40 kDa. Following a similar method used previously (Z. Li, M.S. Thesis, NCSU), except L-Arabinose was increased from 0.02 to 0.2% during induction, NadV-His6 protein was also successfully purified. SDS-PAGE result confirmed a prominent band at ~55.5 kDa.

3.4.1.2 Coupled-enzyme Assay for Measuring NatV and NadV Activities

Synthesis of NAD$^+$ can be measured by fluorimetry (NADH: 340 nm excitation, 460 nm emission) in an assay coupled to alcohol dehydrogenase (ADH), which converts NAD$^+$ to NADH in the presence of ethanol via the reaction Ethanol + NAD$^+$ $\leftrightarrow$ Acetaldehyde + NADH + H$^+$ (5). Establishing reaction conditions and fluorometer settings were a challenge, since different NADH levels affect the instrument gain setting and data collection time. Signal was optimal at a gain of 80; 20 flashes per reading for fluorescence intensity; and 0.2 second positioning delay.

The ADH reaction was first standardized. NAD$^+$ to NADH conversion occurred quickly, reaching a maximum within a couple minutes when 2.5 mM NAD$^+$, 5 units of ADH,
and 3% ethanol were supplied in the reaction. Ethanol was reduced to 1.5% in later reactions for optimal results (24) (19) (20). In contrast to earlier reports (10), the ADH specific activity was most favorable at 25°C, producing 52 µmole NADH/sec/U ADH.

Once the “reporter” activity of ADH was optimized, the NatV NMNATase reaction was tested. The NatV NMNATase reaction was initiated by adding NatV-His6 last to the mixture that contained ADH and all other substrates. The level of NADH can be an indication of NAD⁺ production, since there is 1:1 conversion of NAD⁺→NADH. The specific activity of NatV-His6 was 50 µmole NAD⁺/sec/µg NatV-His6 at 25°C.

NADH fluorescence was also used to measure NMN production, since one molecule of nicotinamide will be converted to NMN, which in the presence of NatV can be converted to NAD⁺ and then to NADH by ADH. The stoichiometry of these conversions, nicotinamide : NMN : NAD⁺ : NADH is 1:1:1:1. The specific activity of NadV was essentially the same at 25°, 30°, and 37°C. The NMN productions were fairly low at 25°C, when less than 50 pmole NadV-His6 were added in the reaction.

Overall, Figure 19 and Figure 20 illustrate that purified NadV-His6 is an active nicotinamide phosphoribosyltransferase that synthesizes NMN from nicotinamide and PRPP. Therefore, the proposed two-reaction pyridine nucleotide (NAD⁺) scavenging pathway (Nam → NMN → NAD⁺) in KVP40 is present and functional.

NatV NMNATase activity was also confirmed by mass spectrometry. However, the result presented a different NatV specific activity. NAD⁺ production per second with 1 ug of NatV detected by mass spectrometry was 7-fold higher (350 µmole) than NAD⁺ production by the NatV-ADH reaction (50 µmole). Clearly, the sensitivity of mass spectrometry, which
directly detects NAD$^+$, is higher than the coupled, ADH-dependent, enzyme assay.

### 3.4.1.3 Analysis of NatV Nudix Hydrolase Activity

NatV has an apparent second domain, Nudix hydrolase, in the C-terminal half of the protein. Two different methods were applied to evaluate substrates for the Nudix activity: an indirect assay by measuring absorbance at 660 nm via a phosphate release assay and a direct assay using mass spectrometry. In the indirect method, the terminal phosphate removed from AMP by alkaline phosphatase is measured using a colorimetric reagent. AMP is a hydrolytic product by Nudix hydrolase reactions on several substrates (6) (14) (26). As different classes of Nudix hydrolases display a wide range of substrates, specificity of NatV Nudix hydrolase from KVP40 for different substrates was tested. By the indirect (Pi release) Nudix hydrolase assay, ADP-ribose, NADH, NAD$^+$, NADPH and FAD were identified as possible substrates for NatV. NADP$^+$, rATP, dATP, rGTP, and AP$_3$A displayed low AMP production per minute. As bovine alkaline phosphatase is active on triphosphates, the APase should have been replaced with inorganic pyrophosphatase when (deoxy) nucleoside triphosphates were used as substrates in the phosphate release assay (26).

The NatV Nudix hydrolase reaction was evaluated directly by mass spectrometry. By direct AMP quantification, ADP-ribose was the best substrate, followed by FAD, NADH and NAD$^+$. NADP$^+$, NADPH, (deoxy) nucleoside triphosphates and AP$_3$A were not substrates for KVP40 NatV Nudix hydrolase. Because of the sensitivity of mass spectrometry, the specific activity of NatV Nudix hydrolase was higher when measured by this method. NatV Nudix
hydrolase specific activity on ADP-ribose detected by spectrophotometry and mass spectrometry was 0.6 μmole AMP/sec/μg NatV-His6. However, preferences for metal ion, other than Mg\(^{2+}\), pH and temperature optima in both indirect and direct assays for the NatV Nudix hydrolase, have not been tested. Overall, ADP-ribose was the best substrate for NatV Nudix hydrolase, followed by NADH and NA D\(^+\), when Mg\(^{2+}\) were used at pH 8.0, 37°C.

The related *Synechocystis* sp. slr0787 bifunctional enzyme, endowed with both NMNATase and Nudix hydrolase activity, has preference for (2′)Phospho-ADP-ribose and ADP-ribose as substrates in the presence of Mn\(^{2+}\) (17). The gene *e.l* (NudE) Nudix hydrolase of T4 bacteriophage has substrate preference of FAD, AP\(_3\)A and ADP-ribose (26). Therefore, the KVP40 NatV Nudix hydrolase appears to be similar, but slightly different from each of these enzymes.

3.4.1.4 NMNATase Activity in KVP40-infected Cells

Using a coupled assay, when ADH converts NAD\(^+\) to fluorescent NADH, the *in vivo* levels of NAD\(^+\) synthesized from NMN could be measured in KVP40-infected cell extracts (G. Edwardsen, NCSU undergraduate research student). KVP40 NatV and NadR both could contribute to the NMNATase activity in the infected cell. *V. parahaemolyticus* cells collected immediately before infection did not show background levels of NAD\(^+\) that were converted to NADH in the coupled assay. The pattern of NMNATase activity through the 40 minute infection cycle was slightly delayed relative to the *natV* mRNA transcription pattern. The peak of NMNPRTase activity (15~20 minutes after infection) was ~5 minutes after the
maximum mRNA level of natV (~10 minutes after infection). Therefore, KVP40-encoded NMNATase activity is clearly evident during phage infection, and higher than that in uninfected cells.

3.4.2 Early Expression of nadV and natV during KVP40 Infection

qRT-PCR is a convenient technology that derives from conventional PCR to analyze the amount of target RNA present in different samples (8) (2). By synthesizing cDNA from RNA in the presence of RNA-dependent DNA polymerase, the copy number of a RNA template can be measured when fluorescence dye binds to double stranded DNA; the fluorescence signal increases as the template is amplified. There are two popular qRT-PCR methods, i) probe-based which is often called TaqMan® PCR and ii) intercalator-based, or more popularly known as the SYBR® Green method. TaqMan® requires both primers and an additional fluorogenic probe which amplify specific DNA sequences that hybridize to the probe sequence. The advantage of the SYBR Green method is that it can be performed with standard primers for each target RNA, allowing several genes to be analyzed in a cost-effective manner. However, the SYBR Green method has some risk of contamination where longer cycle times can lead to artifact amplification. In this study, the SYBR Green qRT-PCR method of Clokie et al. was used to monitor quantification of phage and host gene transcripts during phage KVP40 infection. To attain more accurate and reliable results, three important parameters were evaluated: baseline, threshold and C_T values. During early amplification cycles, fluorescence levels fluctuated because of differences in the reaction mixtures and this
creates a background signal, thus indicating the baseline. The threshold for each reaction was set at one third of the maximum (end) Relative Fluoresce Units (RFU). The Threshold Cycle (C_T) value designates the cycle number where the fluorescence signal is first detected in an amplification reaction (Applied Biosystems tutorial center) (2).

Using this method, the expression patterns of nadV and natV mRNAs were evaluated and compared to the expression of other phage genes. A 40 minute infection period for KVP40 grown on V. parahaemolyticus at 32°C was used based on a previous single-step growth curve (D. Nzella, NCSU undergraduate research student). 16S rRNA (rrn genes) was used as an internal control because it remained constant throughout infection. qRT-PCR analysis clearly revealed expression of nadV and natV during KVP40 infection of V. parahaemolyticus. These genes first appeared 3-5 minutes after infection. Among 7 other phage genes analyzed, td (thymidylate synthase) showed a similar expression pattern as that of nadV and natV. asiA, frd and 55 expression initiated a few minutes later (5-8 minutes) and were therefore designated as ‘delayed early’ genes. Genes that are components of the KVP40 virion particle, 25, wac and 23 were expressed late: 10-15 minutes post infection.

Overall, nadV and natV transcripts appear during a period that for the better characterized phage T4 is typically regarded as the “metabolic phase” of phage gene expression. KVP40 nadV and natV transcription occur when pyridine nucleotide scavenging would be useful for increasing NAD⁺ levels and providing electron carriers for important redox reactions.
Acknowledgements: In addition to those noted in the thesis, I would like to thank Ginger Edwardsen (NCSU Microbiology Honors Research Undergraduate) for conducting NatV and NadV assays on KVP40-infected cells.
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Appendix A. Structures of compounds relevant to the NatV Nudix hydrolase reaction. Appendix A is provided to help illustrate the NatV Nudix hydrolase reaction on each substrate.

Adenosine diphosphate-ribose (ADP-ribose)

Nicotinamide adenine dinucleotide (oxidized, NAD$^+$ and reduced form, NADH)
Flavin adenine dinucleotide (FAD)

Nicotinamide adenine dinucleotide phosphate (NADP⁺)
Nicotinamide adenine dinucleotide phosphate (NADPH)

Adenosine triphosphate (ATP)
Deoxyadenosine triphosphate (dATP)

Guanosine triphosphate (GTP)
Deoxyguanosine triphosphate (dGTP)

Adenosine monophosphate (AMP), Nudix hydrolase product
Diadenosine triphosphate (AP₃A)
Appendix B. Recipe for RNAlater, an RNase-retarding solution useful in qRT-PCR.

This solution stops RNA degradation by penetrating harvested cells. The cells can be stored in this solution for 1 day at 37°C, 1 week at room temperature, 1 month at 4°C or at -20°C or -80°C for longer storage period (Clarke and Amaral). This recipe was obtained from the Department of Chemistry and Biochemistry, Lisbon, Portugal.

Materials: 0.5 M EDTA, pH 8.0; 1 M sodium citrate trisodium salt dehydrate; Ammonium sulphate ([NH₄]₂SO₄) powder; Nuclease free ddH₂O (eg, DEPC-treated); and 1 M sulfuric acid (H₂SO₄)

Method: In a glass beaker,

- 40 ml 0.5 M EDTA (10 mM final conc.)
- 25 ml 1 M sodium citrate (25 mM final conc.)
- 700 g ammonium sulfate (10 M final conc.)
- 935 ml nuclease free ddH₂O

The solution was stored at room temperature, although it can also be refrigerated.
**Appendix C.** Calculation of the number of phage gene transcripts present at different time points. The method is described in detail by Clokie *et al*. This example for *nadV* uses the C\textsubscript{T} values for RNA collected at 1, 3 and 5 min after infection by KVP40.

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<tbody>
<tr>
<td>Time (min)</td>
<td>C\textsubscript{T}</td>
<td>Control</td>
<td>Corrected</td>
<td>Calibrator</td>
<td>(4)-(5)</td>
<td>2\textsuperscript{(6)}</td>
<td>1/(7)</td>
<td>(8)/0.4</td>
<td>(9) x phage</td>
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<tr>
<td>1</td>
<td>34.48</td>
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(1) Time after infection

(2) The average C\textsubscript{T} value of triplicate samples at each time point

(3) The average 16S rRNA C\textsubscript{T} value subtracted from the actual 16S value for each time point.

\[ C\textsubscript{T} \text{ of 16S rRNA at each time point} = C\textsubscript{T} \text{ of 16s rRNA from all time points} \]

(4) \( \Delta C\textsubscript{T} \): C\textsubscript{T} value at each time point was normalized by C\textsubscript{T} – (C\textsubscript{T} of 16S rRNA – average C\textsubscript{T} of 16S rRNA from all time points).

(5) Calibrator: the average C\textsubscript{T} of four KVP40 genomic DNA transcripts (*nadV, g23, frd* and *g55*) amplified by 0.4 ng of genomic DNA (outlier was excluded by Grubb’s Test). Each gene is present in single copy in the KVP40 genome.

(6) \( \Delta \Delta C\textsubscript{T} \): Calibration
(7) $2^{\Delta\Delta C_T}$

(8) Amount of transcript at each time point relative to its internal control, 16S rRNA, and the amount of phage DNA in the calibrator reaction, 0.4 ng

(9) Amount of transcript present relative to 1 ng of KVP40 DNA

(10) Multiplied by the copy number of KVP40 present in 1 ng of DNA, where the size of the KVP40 genome is 244,835 and the average molecular weight of a DNA bp is 660. The number of phage genomes in template (cDNA) can be calculated by molecular mass of the phage multiplied by Avogadro’s number. Thus, the number of phage genomes = \[\frac{2 \times 10^9}{(244,835 \times 660)}\] \(6 \times 10^{23}\) = 7426151.564. Since DNA is double stranded, the number of genomes are measured twice as much.
Appendix D. Examples of raw $C_T$ data for KVP40 genes analyzed using qRT-PCR. Appendix D is provided to help understand the basic approach of using qRT-PCR to analyze phage gene expression. The $C_T$ value for each sample was obtained where a threshold line was positioned at 1/3 of each end RFU. Color indication: Red solid line, before infection; bright green ●, T1 as 1 minute after infection; dark green ▲, T3; turquoise ●, T5; blue ●, T8; pink ■, T10; burgundy ▼, T15; navy solid line, T20; purple ▲, T30; yellow ●, T40.

Early genes:
1) *nadV*
2) $natV$

3) $td$
Delayed early genes:

4) *asiA*

5) *frd*
Late genes:
7) 23
8) wac

9) 25
Internal control, *V. parahaemolyticus* 16S rRNA

KVP40 genomic DNA amplified with *nadV, 23, frd and 55* as calibrator. Duplicate samples per each primer set. Color indication: orange ■, *nadV*; bright green ▲, 23; blue ♦, *frd*; pink ●, 55.