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# Recombinant expression and purification of an ATP-dependent DNA ligase from *Aliivibrio salmonicida*

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ABSTRACT

The genome of the psychrophilic fish-pathogen *Aliivibrio salmonicida* encodes a putative ATP-dependent DNA ligase in addition to a housekeeping NAD-dependent enzyme. In order to study the structure and activity of the ATP dependent ligase *in vitro* we have undertaken its recombinant production and purification from an *Escherichia coli* based expression system.

Expression and purification of this protein presented two significant challenges. First, the gene product was moderately toxic to *E. coli* cells, second it was necessary to remove the large amounts of *E. coli* DNA present in bacterial lysates without contamination of the protein preparation by nucleases which might interfere with future assaying. The toxicity problem was overcome by fusion of the putative ligase to large solubility tags such as maltose-binding protein (MBP) or Glutathione-S-transferase (GST), and DNA was removed by treatment with a nuclease which could be inhibited by reducing agents.

As the *A. salmonicida* ATP-dependent DNA ligase gene encodes a predicted leader peptide, both the full-length and mature forms of the protein were produced. Both possessed ATP-dependent DNA ligase activity, but the truncated form was significantly more active. Here we detail the first reported production, purification and preliminary characterization of active *A. salmonicida* ATP-dependent DNA ligase.



## Introduction

DNA ligases are enzymes which catalyze the formation of a phosphodiester bond between adjacent 5′ $PO_4$ and 3′ OH ends in double-stranded DNA, and are essential for sealing breaks during DNA replication and repair [1]. DNA ligases can be divided into two types based on the nucleotide cofactor they use as an AMP[1] donor: ATP-dependent DNA ligases (EC 6.5.1.1) which are found in eukaryotes and archaea, and NAD-dependent DNA ligases (EC 6.5.1.2) which are found exclusively in bacteria [2]. In addition to their house-keeping NAD-dependent enzymes, many bacteria have one or more ATP-dependent DNA ligases, the evolutionary origin and cellular function of which have not been entirely determined [3]. A number of these accessory enzymes have been biochemically characterized and some are postulated to play a role in DNA repair [4–7] while others are suggested to be involved in competence and DNA uptake [8,9]. The genome of the pathogenic psychrophile *Aliivibrio salmonicida* encodes one such putative ATP-dependent DNA ligase [10].

In order to study its structure and activity *in vitro* we have undertaken the recombinant production and purification of this DNA ligase from an *Escherichia coli* based expression system. Expression and purification of this protein presented two significant challenges. First, the gene product was moderately toxic to *E. coli* cells, second, crude lysates contained a large amount of bacterial DNA which needed to be removed prior to purification without contaminating the ligase protein preparation. Numerous publications have focused on the utility of large fusion partners in increasing protein solubility and expression levels (for example see [11,12]), and comprehensive protocols for the production of MBP fusion constructs are available [13]. However the application of large fusion partners to overcome toxic effects of intracellularly-expressed proteins on the host cells has not been systematically reported to the same extent. In the case of the two Vib-Lig variants described here, the decreased host-cell growth rate with smaller

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[1] *Abbreviations used:* AMP, adenosine monophosphate; Amp, ampicillin; ATP, adenosine triphosphate; BLAST, Basic local alignment search tool; Cam, chloramphenicol; CDS, coding DNA sequence; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GST, glutathione-S-transferase; IPTG, isopropyl β-D-1-thiogalactopyranoside; LB, lysogeny broth; MBP, maltose-binding protein; NAD, nicotinamide adenine dinucleotide; Ni-IMAC, nickel-immobilized affinity chromatography; OD, optical density; RCF, relative centrifugal force; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBE, tris/borate/EDTA buffer; TEV protease, tobacco etch virus protease.

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tags presented a significant loss of efficiency during protein production, even before solubility issues were taken into consideration.

As the *A. salmonicida* gene encodes a predicted leader peptide, both the full-length and mature forms of the protein were produced, and ATP-dependent DNA ligation activity was verified for both constructs. This work represents the first instance of successful production, purification and preliminary characterization of active *A. salmonicida* ATP-dependent DNA ligase.

## Methods

### *Bioinformatics*

The CDS YP_002262821.1 of the *A. salmonicida* genome encodes a 284 amino acid product VSAL_I1366 which is annotated as an ATP-dependent DNA ligase. BLAST homology searches show it has low identity with previously characterized DNA ligases from *Haemophilus influenza* (37%) *Neisseria meningitides* (36%) *Pseudomonas aeruginosa* (30%) and *Mycobacterium tuberculosis* homologues B, C and D (23%, 24%, 27%) [8,9,14,15]. In spite of this low identity, VSAL_I1366 has a number of conserved residues which are involved in DNA ligase activity in homologues, including lysine 52 which is found in the motif I KxDG and is the site of AMP binding. Consistent with the gene annotation, a search of the pfam database [16] identifies two conserved domains: an N-terminal DNA-ligase adenylation domain (pfam01068) from residues 29–202 and a C-terminal oligonucleotide binding domain (cd08040) from residues 216–281.

Analysis with the program SignalP [17] using the 'gram negatives' network produced a strong prediction for a 21 amino acid leader peptide (mean S = 0.726, mean D = 0.692) with a cleavage position between residues Ala 21 and Phe 22 which would direct the enzyme to the periplasm of the bacterial cell [18]. Analysis of the N-terminal sequence hydropathicity [19], along with helix-forming [20] and transmembrane tendencies [21] indicated that this predication falls within a hydrophobic helical sequence Fig. 1. For this reason, during cloning we truncated the polypeptide by a further four amino acids to what we believe is the beginning of the soluble functional domain. The 284 amino-acid full-length protein, denoted FL-Vib-Lig, has a computed molecular mass of 31.7 kDa and estimated pI of 5.68, while the N-terminally truncated protein, denoted TR-Vib-Lig, is 29.1 kDa and has a predicted pI of 5.51.

### *Cloning*

The genes for FL-Vib-Lig and TR-Vib-Lig were both amplified in two stages using Phusion polymerase (New England BioLabs). In the first step, the coding sequence was amplified from a plasmid harboring the full-length *A. salmonicida* ATP DNA ligase gene using reverse primer BK 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCTTAATATTTTTCACGAACC-3′ and either the forward primer FD 5′-GAAAATCTTTATTTTCAAGGTAAAGTATCAACATTATCG-3′ to produce the full-length sequence omitting the codon for the first methionine, or primer FD 5′-GAAAATCTTTATTTTCAAGGTAATACAGTCCCTGTTTCTGTATTG-3′ to produce an N-terminal truncation lacking the first 25 amino acids corresponding the signal peptide. In the second step the PCR products were extended using the primers FD 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCCATCACCATCACCATCACGAAAATCTTTATTTTCAAGGT-3′ and the same reverse primer as in the first PCR reaction to add non-coding attB1 and attB2 sequences for recombination using the Gateway® system (Life Technologies), and coding sequences for an N-terminal 6-His tag followed by a TEV protease cleavage site [22]. These genes were used as substrates in the Gateway® BP reaction with pDONR 221 to produce entry vectors containing the FL- or TR-genes. These entry constructs were transformed into One Shot® TOP10

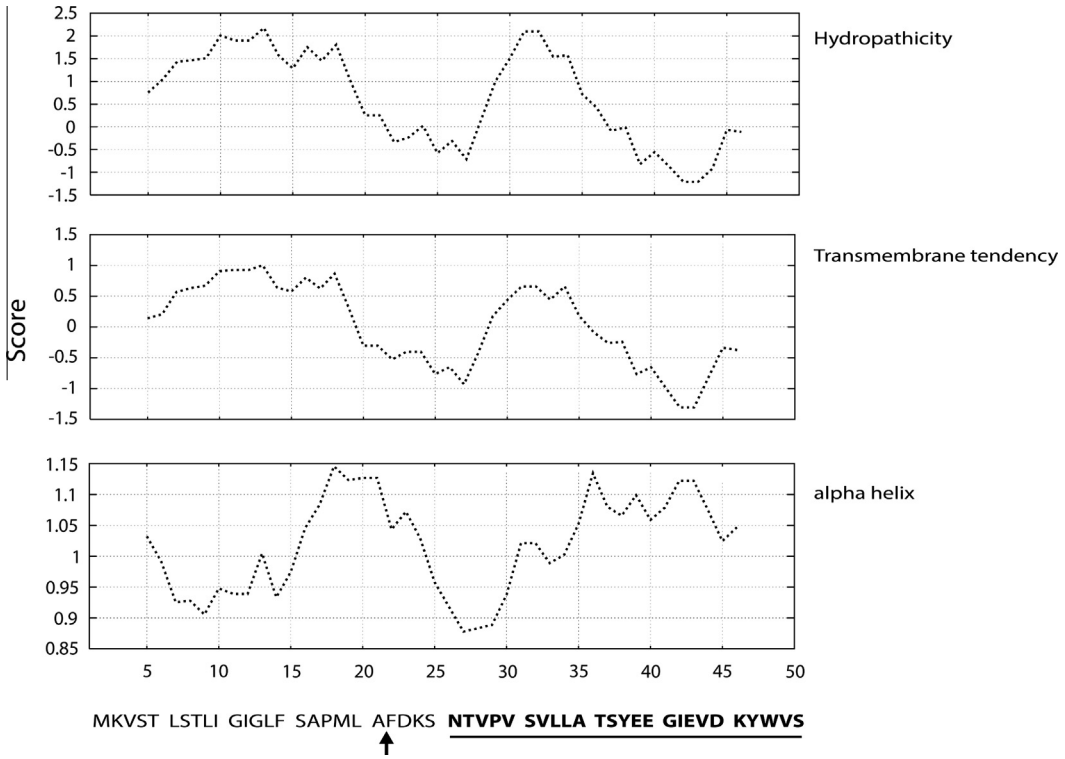


**Fig. 1.** Sequence analysis of the N-terminal region of the putative ATP-dependent DNA ligase from *A. salmonicida*. The arrow indicates the cleavage position suggested by the 'SignalP' program. The bold underlined residues indicate the N-terminal sequence used below for cloning the truncated form.