

# 1 Temperature adaptation of DNA ligases from psychrophilic organisms

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## 6 Abstract

7 DNA ligases operating at low temperatures have potential advantages for use in biotechnological  
8 applications. For this reason, we have characterised the temperature-optima and thermal stabilities  
9 of three minimal Lig E-type ATP-dependant DNA ligase originating from Gram-negative obligate  
10 psychrophilic bacteria. The three ligases, denoted Vib-Lig, Psy-Lig and Par-Lig show a remarkable  
11 range of thermal stabilities and optima, with the first bearing all the hallmarks of a genuinely cold-  
12 adapted enzyme, while the latter two have activity and stability profiles more typical of mesophilic  
13 proteins. A comparative approach based on sequence comparison and homology modelling indicates  
14 that the cold-adapted features of Vib-Lig may be ascribed to differences in surface charge rather  
15 than increased local or global flexibility: which is consistent with the contemporary emerging  
16 paradigm of the physical basis of cold adaptation of enzymes.

## 17 Keywords

18 ATP-dependent DNA ligase; psychrophile; enzyme activity; temperature optima

## 19 Introduction

20 DNA ligases are DNA-joining enzymes essential for survival of all organisms, due to their critical roles  
21 in DNA replication and repair. Using ATP or NAD<sup>+</sup> as a cofactor, DNA ligases catalyze the formation of  
22 a phosphodiester bond between the 5' phosphate of one DNA strand and the hydroxyl group at the  
23 3' end of the other DNA strand, producing an intact sugar-phosphate backbone. The enzymatic  
24 reaction mechanism can be divided into three nucleotidyltransfer steps (Ellenberger and Tomkinson  
25 2008); the first involves the activation of the enzyme through a nucleophilic attack by a lysine residue  
26 to the adenosine cofactor ATP or NAD<sup>+</sup>, releasing nicotinamide mononucleotide for NAD-dependent  
27 ligases (NDLs) or di-phosphate in the case of ATP-dependent ligases (ADLs). Next, the nucleophilic 5'-  
28 phosphate of the DNA attacks the phosphoramidate bond to form an adenylated-DNA intermediate.  
29 The final step involves attack of the 3'-nucleophilic hydroxyl group on the new pyrophosphate bond,  
30 forming a phosphodiester bond between the 5' and the 3' position of the DNA and releasing the  
31 AMP. All three chemical steps depend on a divalent cation, which is usually Mg<sup>2+</sup> or in some cases  
32 Mn<sup>2+</sup>.

33 DNA ligases are divided into two main classes based on the cofactor required in step 1 of the  
34 enzymatic reaction. The ADLs use ATP and are found in all phylogenetic kingdoms, with eukaryotes,  
35 archaea and many viruses possessing at least one ADL that is essential for DNA replication (by joining  
36 Okazaki fragments), and some encode multiple forms with dedicated roles in DNA repair (Ellenberger  
37 and Tomkinson 2008). NDLs meanwhile are found almost exclusively in bacteria where they function

38 in both replication and repair (Dwivedi et al. 2008; Wilkinson et al. 2001). In the cases where  
39 accessory ADLs are identified in bacteria, it is always in addition to the essential NDLs (Pitcher et al.  
40 2007b).

41 Since the first X-ray crystal structure of an ADL was solved two decades ago from bacteriophage T7  
42 (Subramanya et al. 1996), numerous structural analyses of bacterial, archaeal and eukaryotic ADLs  
43 have followed (Nishida et al. 2006; Pascal et al. 2004) (Kim et al. 2009; Nishida et al. 2006; Pascal et  
44 al. 2004; Petrova et al. 2012) (Akey et al. 2006; Kaminski et al. 2018; Pascal et al. 2006; Shi et al.  
45 2018; Williamson et al. 2018; Williamson et al. 2014), and the wide variety of domains and gene  
46 arrangements between the different classes of ligases has become evident. Crystallographic studies  
47 of bacteriophage T7 (Doherty and Wigley 1999; Subramanya et al. 1996) revealed a common core  
48 architecture of two essential catalytic core domains: the adenylation domain (AD) directly involved in  
49 catalysis and the site of step 1 enzyme-adenylation, and the smaller oligonucleotide/oligosaccharide  
50 binding domain (OB) that is also required for activity (Doherty and Suh 2000; Doherty and Wigley  
51 1999). These core catalytic domains include six conserved motifs (I, III, IIIa, IV, V, and VI) which are  
52 involved in one or more steps of the ligation pathway (Shuman 2009). The AD- and OB domains are  
53 connected by a flexible linker that allows them to reorient during DNA binding. An additional N-  
54 terminal DNA binding domain has been described in the larger ADLs active in DNA replication in  
55 Eukarya and Archaea, and additional enzymatic domains with end-repair functions are appended to  
56 the large LigD enzymes involved in bacterial non-homologous end joining (Pitcher et al. 2007a). The  
57 Lig E group of ADLs, found predominantly in Gammaproteobacteria, have no additional DNA-binding  
58 of enzymatic domains, and may serve as a model for the minimal functional unit of the ATP-  
59 dependent ligases. The ADL from the marine psychrophile *Psychromonas sp.* strain SP041 (Psy-Lig) is  
60 the smallest DNA ligase that has been structurally studied, being 41 residues shorter than the  
61 minimal ChIV-Lig protein (Williamson et al. 2014). Recent structure-function analysis of Psy-Lig and  
62 the closely related Ame-Lig demonstrated a novel mode of ligase engagement with its DNA substrate  
63 that relies on well-ordered side-chain contacts on the surface of the conserved domains, rather than  
64 re-ordering of flexible loop regions to achieve encirclement of the DNA duplex as was previously  
65 observed for minimal viral ligases (Nair et al. 2007; Williamson et al. 2018). All Lig E-type ADLs have  
66 strong predictions for N-terminal leader sequences proposed to direct them to the periplasm.  
67 Proposed biological functions of such secreted ligases include competence and DNA uptake in the  
68 periplasm (Magnet and Blanchard 2004), and the demonstrated increase in activity and solubility  
69 when this predicted leader was not included in recombinantly-produced *Aliivibrio salmonicida*  
70 (hereafter referred to as Vib-Lig) supports such signal processing (Williamson and Pedersen 2014).

71 In the present study we have characterised the temperature-optima and thermal stability of Psy-Lig  
72 and Vib-Lig, both of which originate from obligate psychrophiles, along with a third homolog from  
73 *Pseudoalteromonas artica* (hereafter Par-Lig), isolated from sandy beach sediment on the Arctic  
74 island of Svalbard (Al Khudary et al. 2008). In order to understand structural determinants for low-  
75 temperature activity, possible psychrophilic properties of these enzymes were studied. This builds on  
76 previous work by Georlette *et al.* who conducted biophysical analyses and biochemical comparisons  
77 of larger, more complex NDLs from species spanning a range of growth temperature optima  
78 (Georlette et al. 2003; Georlette et al. 2000).

79 Living and thriving at low temperatures requires that both enzyme kinetics and protein stability are  
80 adapted accordingly. It is now widely accepted that structural differences between cold-active

81 enzymes and their mesophilic counterparts enable high specific activity at low temperatures, with a  
82 lower energy cost (D'Amico et al. 2002; Feller 2003; Struvay and Feller 2012). The physical origin of  
83 decreased temperature optima imparted by these structural changes are an active area of  
84 contemporary investigation (Åqvist et al. 2017; Arcus et al. 2016; Isaksen et al. 2016; Saavedra et al.  
85 2018; van der Kamp et al. 2018), but it is generally observed that improved catalytic efficiency is  
86 accompanied by a reduced thermal stability and weaker substrate affinity, compared to  
87 thermophiles and mesophiles at the opposite end of the temperature scale (Struvay and Feller 2012).  
88 For this reason we have also carried out *in silico* comparisons of these Arctic-derived ADLs with  
89 mesophilic-derived counterparts from human pathogens.

90 DNA ligases adapted to low temperatures offer novel potential advantages for use of these enzymes  
91 in biotechnological applications. Recently, the thermolability of a cold adapted DNA ligase was used  
92 to develop a novel temperature-sensitive vaccine for tularemia (Duplantis et al. 2011), showing great  
93 potential in the biomedical science and other applications where bacterial growth control is crucial.  
94 Further, the enzymatic activity performed by DNA ligases in DNA replication and repair makes them  
95 useful tools in molecular biology and biotechnology applications, such as genetic engineering and  
96 next-generation DNA sequencing technologies (Chambers and Patrick 2015; Shuman 2009; Tanabe et  
97 al. 2015). Cold-adapted enzymes have a potential advantage over mesophilic homologs by increasing  
98 yields of product at low temperatures, while suppressing contaminating nuclease activity. Finally,  
99 should the cold-active ligases be highly active, protocols may be carried out with smaller amounts of  
100 enzyme, due to better activity rates. In particular, short base-pair overhangs, i.e. 'sticky ends'  
101 generated by many restriction enzymes will be stabilized due to the low melting temperature of  
102 short tracts of base-pairing involved. For these reasons, improving our understanding of temperature  
103 adaptation and identification of psychrophilic traits that could be used directly, or reverse-  
104 engineered into commercial ligase scaffolds has important biotechnological applications.

## 105 Methods

### 106 Protein expression and purification

107 ADLs from *Psychromonas spp.* strain SP041 (Psy-Lig) and *Aliivibrio salmonicida* (Vib-Lig) were  
108 expressed and purified as described previously (Williamson and Pedersen 2014; Williamson et al.  
109 2014). The gene encoding the Lig E-type ADL from *Pseudoalteromonas artica* (WP\_010555135; Par-  
110 Lig), without the leader peptide, was synthesized by *Life Technologies* as the mature His-tagged, TEV-  
111 cleavable form with codon optimization for *E. coli* and supplied in the donor vector pDONR221.  
112 Transfer to the PHMGWA vector was done using Gateway® cloning (Thermo Fisher), and all steps  
113 including expression of the MBP-fusion, purification and tag removal were carried out as described  
114 for Psy-Lig and Vib-Lig.

### 115 Enzyme assays

116 Gel-based endpoint assays were carried out as described previously using 20 nt + 20 nt oligomers to  
117 form 40 nt product (Williamson et al. 2018; Williamson et al. 2014). Details of substrate preparation  
118 are given in Table S1. Reactions contained 80 nM substrate, 1 mM ATP, 10 mM MgCl<sub>2</sub>, 10 mM DTT,  
119 50 mM NaCl, 50 mM Tris-HCl pH 8.0. Enzymatic activity was detected by conversion of the FAM-  
120 labeled 20 nt substrate oligonucleotide into a 40 nt product, resolved by denaturing electrophoresis,  
121 detected by fluorescence on a Pharos FX Plus imager (Biorad) and quantified by band intensity using

122 the software Image J (Schneider et al. 2012). The extent of ligation activity was calculated from the  
123 ratio of these band and expressed as a percentage. The temperature dependence of ligase activity  
124 was investigated by assaying for 15 min at temperatures between 5 °C to 55 °C for nicked substrates  
125 and 5 °C to 35 °C for cohesive substrates. Reactions were allowed to equilibrate for 1 min to the  
126 assay temperature, and then the assay was started by addition of the enzyme.

### 127 Differential scanning calorimetry

128 Differential scanning calorimetry (DSC) experiments were carried out using an N-DSC III differential  
129 scanning calorimeter (Calorimetry Sciences Corporation). Purified ligases with concentrations of 1-2  
130 mg ml<sup>-1</sup> were extensively dialyzed against 50 mM HEPES pH 8.0, 100 mM NaCl to ensure complete  
131 equilibration. The enzymes were filtered through a 0.2 µm syringe filter (Millipore, Billerica, USA) and  
132 degassed for approximately 15 min before being loaded into the sample cell. The dialysis buffer was  
133 used as reference for baseline subtraction. Data analysis was performed using the program  
134 NanoAnalyse 2.4 (TA instruments). For each protein sample scanned the corresponding buffer  
135 baseline was subtracted, and the data were normalized to the molar protein concentration  
136 calculated from the absorbance at 280 nm after dialysis and filtration. The calorimetric enthalpy was  
137 determined directly from the experimental data, and a theoretical two-state model was fitted using  
138 the routines provided in the program for determination of the van 't Hoff enthalpy.

### 139 Thermofluor

140 Thermal denaturation of the purified ADLs with different buffers were examined by the thermofluor  
141 assay as described previously (Ericsson et al. 2006). Briefly, 5 µl of protein (1.0-1.5 mg ml<sup>-1</sup>) was  
142 mixed with 1 µl of 300 x Sypro-Orange, 12.5 µl of 50 mM HEPES pH 8.0, 200 mM NaCl, added to the  
143 wells of a 96-well PCR plate (Bio-Rad) and sealed with Microseal® 'B' Adhesive Seals from Bio-Rad.  
144 Melting curves were recorded from 20 °C to 90 °C in increments of 0.3°C per sec using a MiniOpticon  
145 Real-Time PCR System with both FAM and HEX dye channels selected. T<sub>m</sub> was determined using the  
146 supplied instrument software and monitoring the fluoresce of the HEX channel.

### 147 Sequence comparison

148 The amino acid sequences of Par-Lig, Psy-Lig and Vib-Lig, were aligned with the Lig E sequence from  
149 *Vibrio cholera* (Vch-Lig; gi|147674166). N-terminal leader sequences were predicted using SignalP  
150 4.1 and omitted from further analyses (<http://www.cbs.dtu.dk/services/SignalP/>)(Petersen et al.  
151 2011). The ClustalW alignment tool in BioEdit was used to determine sequence identities and  
152 similarities. Conserved domains were analysed by Pfam protein families database at EMBL-EBI  
153 (<http://pfam.xfam.org>).

### 154 Homology modelling and analysis

155 Homology models of Vib-Lig, Par-Lig and Vch-Lig were built based on the deposited crystal structure  
156 of Psy-Lig (4D05; (Williamson et al. 2014)). The sequences were uploaded to the Swiss-Model  
157 homology modeling server (Biasini et al. 2014). The A-chain of the deposited structure of Psy-Lig was  
158 selected as a modeling template for all modeled structure as it has overall superior quality than the  
159 B-chain (with lower overall B-factor and amino acid residues generally better defined in electron  
160 density).

161 HBPLUS Hydrogen Bond Calculator v 3.2 (McDonald and Thornton 1994) was used to calculate  
162 hydrogen bonds in all PDB-files. The hydrogen bonds included were those fulfilling the criteria for

163 parameters donor (D), acceptor (A), acceptor antecedents (AA) and calculated hydrogen (H):  
164 maximum distance for D-A, 3.5 Å and H-A, 2.5 Å; minimum angle for D-H-A, D-A-AA and H-A-AA of  
165 90°. Ion-pair-interactions were investigated using the WHAT IF Web Interface  
166 (<http://swift.cmbi.ru.nl/servers/html/index.html>) (Vriend 1990), where interatomic distances  
167 between the side-chains of the negatively charged Asp and Glu, and the positively charged Arg, Lys  
168 and His were tabulated with respect to being <4 Å and <6 Å. The APBS plugin in Pymol was used to  
169 estimate electrostatic surface potentials (Dolinsky et al. 2007).

## 170 Results

### 171 Temperature optimum and thermal stability

172 The aim of this study was to understand the determinants of low-temperature adaptation among  
173 DNA ligases. We chose to investigate the temperature optimum and thermal stability of Lig E ADLs  
174 from *Psychromonas spp.* strain SP041, *Aliivibrio salmonicida*, and *Pseudoalteromonas artica*,  
175 delineated Psy-Lig, Vib-Lig and Par-Lig respectively as these represent psychrophilic species of  
176 bacteria isolated from a consistently low-temperature environments (Al Khudary et al. 2008; Egidius  
177 et al. 1986) (**Error! Reference source not found.**).

178 To analyze the temperature optima for ligase activity, gel-based endpoint assays were performed,  
179 both with single nicked and overhanging substrates. Nick sealing activity was measured by  
180 temperature intervals of 5°C, ranging from 5 to 60 °C, or until the activity was abolished. For ligation  
181 of single-nicked substrates (Figure 1a), there is a sharp peak of more than 50% ligation activity at  
182 around 20°C for Vib-Lig, quickly declining to 10% activity at 30°C, whereas the activity of Psy-Lig and  
183 Par-Lig increases with temperature from 15 °C up to an optimum of 35-40°C, above which a sharp  
184 decline is observed. Although all ligases were cloned from psychrophilic organisms with similar  
185 growth temperatures, the  $T_{opt}$  of their ligases for nicked substrates are different (Table 1).

186 The characterized ligases Psy-Lig, Par-Lig and Vib-lig show a similar and relatively broad temperature  
187 optimum on the overhang substrate tested, with approximately 60-80 % ligation activity from 5°C to  
188 25-30°C, followed by a sharp decline at higher temperatures. As they all show better activity on  
189 overhang breaks at lower temperatures, we suggest that substrate stability rather than enzyme  
190 activity is the driving feature here. However, the enzymatic reaction will work very slowly at the low  
191 temperature, requiring a longer incubation time.

192 DSC experiments were performed to obtain a complete thermodynamic profile of the protein  
193 unfolding process of Psy-Lig, Par-Lig and Vib-Lig. The melting temperature ( $T_m$ ) was estimated to be  
194 significantly lower for Vib-Lig, 30.7 °C, compared to Psy-Lig and Par-Lig with a  $T_m$  of 46.0 °C and 53.7  
195 °C, respectively (Figure 2a). All three ligases measured show a ratio > 1 between the van 't Hoff  
196 enthalpy derived from fitting a two-state model, and the calorimetric enthalpy, derived by  
197 integration of the area under the excess heat capacity. Such temperature profile indicates that  
198 unfolding proceeds as a higher order oligomer; however, the irreversibility of the unfolding transition  
199 precluded detailed thermodynamic analysis.

200 The thermal stability in various buffer systems was measured by a thermofluor assay to confirm the  
201 DSC results and exclude the possibility that low thermal stability of observed for Vib-Lig is caused by  
202 non-ideal buffer conditions as it has a significantly lower pI (predicted to be 5.5) relative to Psy-Lig  
203 and Par-Lig (both greater than 9.0). Thermofluor data (Figure 2b) suggest that stability of the various

204 ligases does not vary between pHs 6.5 and 9, with the exception of Psy-Lig which is extremely  
205 unstable in phosphate buffer at pH 7.0. Otherwise, Psy-Lig shows stability up to 46 °C and Par-Lig up  
206 to 53 °C, which is in line with DSC unfolding temperature. Also consistent with DSC data, Vib-Lig  
207 shows a lower thermal stability relative to Psy-Lig and Par-Lig with a maximum at 23 °C in all buffers  
208 down to pH 6.5. Below this, no transition could be observed, indicating that Vib-Lig was already  
209 unfolded.

## 210 Sequence comparison

211 Cold-active enzymes may combine rigidity and stability with a high level of flexibility. To gain further  
212 insight into the activity/stability/flexibility relationship and cold adaptation, interesting sequence and  
213 structural differences were identified by sequence alignments and homology modelling.

214 The enzymes studied are of similar size and share all properties common to minimal ADLs but exhibit  
215 different temperature optima and stabilities. A structure-based sequence alignment was generated  
216 (Figure 3). Lig E from *V. cholera* (Vch-Lig) was included as this human pathogen has growth  
217 temperature between 20 and 45 °C and is unable to survive at 4 °C for extended periods of time  
218 (Martinez et al. 2010). Pairwise comparison of the three experimentally-examined Lig Es together  
219 with Vch-Lig show that all sequence pairs have identities in the 40-49% range. Consistent with both  
220 enzymes deriving from members of the genus *Vibrio*, Vib-Lig and Vch-Lig share the highest homology  
221 in terms of sequence identity (48.4 %), although they are adapted to different habitats and  
222 temperatures; thus Vch-Lig represents a phylogenetically-related mesophilic homolog of Vib-Lig. All  
223 four Lig Es contain the conserved nucleotidyltransferase family motifs I-VI and align with very few  
224 insertions or deletions, giving high confidence in placement of secondary structural elements by  
225 homology modeling (described below). Further, the sequence alignment revealed high conservation  
226 of amino acids involved in substrate binding, metal binding and enzymatic activity.

227 Several studies have indicated increased occurrence of some residues in cold-adapted proteins and  
228 decreased frequency of others, which has been rationalized by physical properties of their sidechains  
229 influencing flexibility and stability of the protein. This includes fewer salt bridges, fewer hydrogen  
230 bonds, a lower content of proline residues, a reduced Arg/(Arg + Lys) ratio, lower (Leu + Ile)/(Leu + Ile  
231 + Val) ratio and increased glycine content (Aghajari et al. 1998; Collins et al. 2005; Huston et al. 2004;  
232 Metpally and Reddy 2009; Russell et al. 1998; Saavedra et al. 2018). For this reason, we compared  
233 the amino acid content of the four proteins; however most classic sequence 'traits' of cold-  
234 adaptation, including increased glycine, decreased proline and less-packed hydrophobic core, were  
235 not apparent in Vib-Lig. Instead, higher sequence conservation appeared to be with the more  
236 phylogenetically-related Vch-Lig than the other psychrophile-derived ADLs. For example, a lower  
237 number of Gly residues is often pinpointed as a typical cold adapted trait, however this did not  
238 correlate with thermal stability of these ADLs, and most Gly residues are conserved, especially  
239 between the psychrophilic Vib-Lig and the mesophilic Vch-Lig (Table 2). Likewise, decreased Pro  
240 content has also been related to cold-adaptation (Wallon et al. 1997; Zhao et al. 2010), but as Vch-  
241 Lig has fewer Pro than Vib-Lig (11 versus 13) Pro content is not an evident factor.

242 Another 'typical' feature of cold-adapted enzymes is a decreased number of Arg residues, which may  
243 increase stability through its capability to form hydrogen bonds and salt bridges (Aittaleb et al. 1997).  
244 In line with this, we observed the highest Arg count in the presumably mesophilic Vch-Lig (Table 2).  
245 The number of Arg is significantly lower for Vib-Lig (11), Psy-Lig (12) and Par-Lig (11) compared to

246 Vch-Lig (18). This is also reflected by the ratio Arg/(Lys+Arg) per residue, which is 0.53 in Vch-Lig  
247 compared to 0.40, 0.39 and 0.39 in Psy-Lig, Vib-Lig and Par-Lig, respectively, also supporting an  
248 overall better stability of the mesophilic molecule. Arg can contribute in more interactions with  
249 surrounding amino acids than lysine. However, Arg may also interact with water on the surface.  
250 Interestingly, the multiple sequence alignment (Figure 3) shows that Arg in Vch-Lig are frequently  
251 substituted with hydrophobic residues in Vib-Lig.

## 252 Homology modeling and comparison to the crystal structure of Psy-Lig

253 To identify positions in the three-dimensional ligase structure where relevant amino acid  
254 substitutions occurred, homology models of Par-Lig, Vib-Lig and Vch-Lig were built based on the  
255 deposited structure of Psy-Lig 4D05; (Williamson et al. 2014)) . Increased local and/or global  
256 flexibility can be achieved by destabilization of the structure through a reduction in intramolecular  
257 forces such as salt-bridges, ion-pair networks, hydrogen bonds and aromatic interaction, and  
258 increased length of loop regions (Davail et al. 1994; Feller 2003; Russell 2000). Hydrogen bond  
259 analysis **Error! Reference source not found.** shows that Vib-Lig is possibly destabilized by fewer  
260 hydrogen bonds per residue in total, compared to Par-Lig and Psy-Lig (0.715/0.778/0.759). In  
261 comparison, the mesophilic Vch-Lig has the highest ratio of hydrogen bonds per residue (0.816). It is  
262 interesting to note that the ratios correlate well with the measured melting temperatures Vib-Lig,  
263 Psy-Lig and Par-Lig with low ratios giving low melting temperatures. In particular, the number of side-  
264 chain to main-chain hydrogen bonds is lower for the cold-adapted Vib-Lig.

265 Examination of the structural models also revealed that the substituted arginines described in the  
266 preceding section are generally located on the surface, thus introducing hydrophobic surface patches  
267 in Vib-Lig (Figure 4). Calculations by POPS (Parameter OPTimsed Surfaces (Fraternali and Cavallo 2002))  
268 showed that the overall total area of exposed hydrophobic residues were similar among all ligases,  
269 thus, unique exposed hydrophobic patches in Vib-Lig appear to be local. Interesting Arg substitutions  
270 in Vib-Lig compared to Vch-Lig include Arg95 to Ala90, Arg167 to Ile162, Arg193 to Thr188, Arg209 to  
271 Gln204 and Arg257 to Ala252 (Figure 4). For Par-Lig and Psy-Lig, three of these Arg are substituted with  
272 Leu/Lys. The percentage of hydrophobic residues is slightly higher for Vib-Lig (42,80%) and Psy-Lig  
273 (41,25%) compared to Par-Lig (39,25%) and Vch-Lig (39,69%), possibly reflecting the substitutions of  
274 polar residues with hydrophobic residues on the surface compared to Vch-Lig. In combination, the  
275 elevated number of hydrophobic residues described above, the unique local hydrophobic surface  
276 patches and the lower number of Arg, may impart local flexibility to the Vib-Lig structure compared to  
277 its mesophilic counterpart Vch-Lig.

## 278 Electrostatic surface potential

279 Some cold adapted enzymes feature an overall excess of negative charges at the surface of the  
280 protein, with a pI frequently more acidic than that of their mesophilic homologues(Feller 2003; Leiros  
281 et al. 1999; Russell 2000). Higher frequency or patches of acidic residues on the surface may increase  
282 solvent interactions and thereby lead to an overall destabilization of the enzyme by charge-charge  
283 repulsion, observed in cold-adapted trypsin and  $\beta$ -lactamase (Feller 2003; Leiros et al. 1999). The  
284 calculated pI of 5.3 for Vib-Lig is significantly more acidic compared to its counterparts, and also  
285 correlates with the substitution of basic arginine residues at the surface with hydrophobic amino  
286 acids. Further examination of the charge distribution on the surface of the Vib-Lig model (Figure 5)  
287 indicates that the DNA-binding faces of Vib-Lig remain positively charged as seen for structures of  
288 other Lig Es, while surfaces not involved in DNA binding are more positively charged compared with

289 the more thermostable Psy-Lig. This suggests that charges in the binding surfaces of Vib-Lig are  
290 conserved and the majority of variation located in distant areas of the protein.

### 291 Conservation of Active site and DNA-binding surface

292 It is often suggested that low-temperature adaptation of enzymes is driven by increased local  
293 flexibility at the active site (D'Amico et al. 2002; D'Amico et al. 2006; Struvay and Feller 2012),  
294 therefore we examined three key areas of the Vib-Lig enzyme that are essential for activity: the  
295 region surrounding the AMP-binding pocket where the enzyme is covalently adenylated in the first  
296 step of the ligase reaction, the inter-domain linker region which undergoes significant structural  
297 changes during the catalytic cycle and the surfaces of the adenylation (AD-) and oligonucleotide-  
298 binding (OB-) domains that are in contact with double-strand DNA during nick-sealing. Our  
299 comparisons reveal that the active site is strictly conserved, except for Lys 41 in Psy-Lig which is  
300 replaced by the chemically-similar Arg in the other three ADLs (Fig 3, supplementary figure S1a). The  
301 sequence alignment shows that the flexible linker regions connecting the two core domains are  
302 similar, preserving the hydrogen bonding pattern observed in Psy-Lig, with the exception of Par-Lig  
303 where the equivalent of Lys 176 (Psy-Lig) is replaced by Pro (Fig 3, supplementary figure S1b). Lig E-  
304 type ligases efficiently ligate DNA breaks without any additional DNA-binding domains or large  
305 flexible loop regions, instead using interactions with shorter highly structured motifs and specific  
306 charged residues found on the DNA-binding surface of the core catalytic domains (Williamson et al.  
307 2018; Williamson et al. 2014). In general these motifs are well conserved between the three variants,  
308 consistent with both the equivalent positively-charged DNA binding surfaces of Vib-Lig and Psy-Lig  
309 and previous observations of consensus between Lig Es in this region (Fig 5) (Williamson et al. 2018).

### 310 Discussion

311 In this study, biochemical and biophysical characteristics of ATP-Dependent Ligases (ADLs) from  
312 psychrophilic organisms were analyzed in an attempt to identify typical cold-adaptation features.  
313 Vib-Lig, originating from the psychrophilic fish pathogen *Aliivibrio salmonicida* which has a growth  
314 range of 1-22 °C and an optimum of 15 °C (Egidius et al. 1986) exhibits classical features of cold  
315 adaptation including a low temperature optimum of activity compared to homologous enzymes and  
316 decreased thermostability. In contrast, despite being derived from psychrophilic organisms, the Psy-  
317 Lig and Par-Lig enzymes are not themselves cold-adapted as both have temperature optima in the  
318 range of 35-40 °C and unfolding temperatures greater than 45 °C. This has been observed for many  
319 psychrophile-derived enzymes such as L-haloacid dehalogenase from *Psychromonas ingrahamii*,  
320 alcohol dehydrogenase of *Flavobacterium frigidimarum* and KUC-12-keto acid decarboxylases derived  
321 from *Psychrobacter*, where the individual enzymes remain active and stable at temperatures well  
322 above the survival limit of the host organism (Kazuoka et al. 2007; Novak et al. 2013; Wei et al.  
323 2013). The simplest rationale in the case of the ADLs is that although the temperature optimum is  
324 relatively high, the 30 – 40 % activity recorded below 15 °C is sufficient for the biological purposes of  
325 the bacterium in its native environment, although the effects of different conditions on *in vivo*  
326 activity are also possible.

327 During ligation of double-strand breaks with cohesive ends, low temperature is an advantage to  
328 stabilize base-pairing between short stretches of complementary nucleotides at the break site. This  
329 must be balanced against decreased enzyme activity at lower temperatures. Lower temperatures  
330 allow DNA overhangs to base-pair and remain annealed long enough for the ligase to join them, at the



331 expense of reduced ligase activity. This is directly observed in the present study during ligation of  
332 substrates with 4nt overhangs as optimal activities are shifted to lower temperatures for all three  
333 enzymes measured, despite of their individual  $T_{opt}$  varying when measured with a nicked substrate.

334 The Lig E enzymes compared in our study have moderate sequence identities (40-50%) and likely  
335 highly similar structures. The analyses performed indicated some sequence differences that  
336 potentially lower  $T_{opt}$  of Vib-Lig relative to homologs. One difficulty in such comparative analyses is  
337 distinguishing between substitutions imparting psychrophilicity and those that have occurred  
338 through genetic drift. To exclude possible false-positive findings based on phylogenetic resemblance,  
339 we included Lig E from *V. cholera* in our sequence comparison as previous phylogenomic studies  
340 have placed this close to Vib-Lig in evolutionary terms (Williamson et al. 2016), thus representing a  
341 genus-related but mesophilic organism. Coming from a mesophilic human pathogen, Vch-Lig is not  
342 anticipated to exhibit cold-adapted characteristics. The major differences in Vib-Lig appear to be in  
343 non-DNA-binding surface exposed residues. Arginines are generally located on the surface of Psy-Lig,  
344 Par-Lig and Vch-Lig, and substitution of these positions introduces hydrophobic or uncharged surface  
345 patches in Vib-Lig. This is consistent with the investigation of three structurally homologous NAD<sup>+</sup>-  
346 dependent DNA ligases (NDLs) adapted to different temperatures, where specific surface areas  
347 revealed a significant increase of exposed hydrophobic residues to solvent, in contrast to a more  
348 hydrophilic and charged surface area in thermophiles (Georgette et al. 2003), indicating an entropy-  
349 driven destabilization of the protein structure. Likewise, replacements of lysine with arginine in the  
350 psychrophilic  $\alpha$ -amylase from *Pseudoalteromonas haloplanktis* resulted in a more stabilized enzyme  
351 with mesophilic properties, demonstrating the relevance of arginine content in cold adaptation  
352 (Siddiqui et al. 2006). It is interesting to note that these substitutions in Vib-Lig are unevenly  
353 distributed between the two domains with only two occurring in the larger catalytic adenylation  
354 domain (approximately 170 residues), and four on the smaller oligonucleotide domain  
355 (approximately 80 residues). Recent work demonstrated that substitutions increasing flexibility in  
356 different domains of adenylate kinase gave rise to different temperature effects on substrate binding  
357 and catalysis (Saavedra et al. 2018). As with DNA ligases, adenylate kinase activity involves  
358 coordinated reorientations between discrete protein domains, and it is interesting to consider  
359 whether this distribution reflects tuning of the oligonucleotide binding domain for DNA binding/  
360 product release rather than catalysis, as the former is are the rate limiting processes (Bauer et al.  
361 2017; Lohman et al. 2011).

362 Calculations of the electrostatic surface potential revealed that the cold-active Vib-Lig displays a  
363 positively charged surface near the active site and on the binding face of the OB-domain, which is  
364 important for binding of the negatively charged DNA substrate (Fig 5), despite its overall more acidic  
365 pI. Similar results were observed for the cold-adapted uracil-DNA *N*-glycosylase (cUNG) from Atlantic  
366 cod (Leiros et al. 2003), indicating increased affinity for the negatively charged DNA compared with  
367 mesophile homologues. The number and nature of residues around the active site are conserved  
368 among the homologous ADLs adapted to different temperatures, suggesting that local cold adapted  
369 residues are not directly involved in catalysis, but influence flexibility indirectly at some distance  
370 apart. The psychrophilic Vib-Lig is further characterized by a decreased number of hydrogen bonds,  
371 which correlates with an increase in overall flexibility of the enzyme and affects protein water  
372 surface interactions.

373 Although the decreased temperature optima of psychrophile-derived enzymes is commonly  
374 attributed to an increase in flexibility, either global or local, which causes a concomitant lowering of  
375 thermal stability, (Smalas et al. 2000) (D'Amico et al. 2002; Feller 2003; Struvay and Feller 2012)  
376 many enzymes are inactivated by temperatures below those inducing denaturation. A comparison of  
377 the NDL from the psychrophile *Pseudoalteromonas haloplanktis* with that of mesophilic NDL of *E. coli*  
378 and the thermophilic NDL of *Thermus scotoductus* found that structural differences imparted a  
379 temperature optimum of 18 °C, compared to 30 °C for *E. coli* and more than 60 °C for *T. scotoductus*  
380 (Georlette et al. 2000). This is accompanied by a decrease in  $T_m$  in the *P. haloplanktis* enzyme (33 °C)  
381 compared to the ones from *E. coli* (54 °C) and *T. scotoductus* (95-101 °C) (Georlette et al. 2003). The  
382 temperature optimum for activity of the *E. coli* NDL corresponds to the beginning of the thermal  
383 unfolding. *P. haloplanktis* NDL, however, shows a different link between activity and thermal  
384 adaptation; optimal activity is reached 10 °C before unfolding and the enzyme is inactivated at the  
385 beginning of the unfolding transition. A similar behaviour is observed for the activity and stability of  
386 Vib-Lig, Psy-Lig and Par-Lig, where a decrease in activity above  $T_{opt}$  is observed in the absence of  
387 denaturation/unfolding. Recently, new paradigms have been suggested to explain this behaviour, as  
388 the classical (two-state) model is limited to enzymes where increased catalytic activity is directly  
389 followed by thermal inactivation. These include macromolecular rate theory (MMRT), which provides  
390 a rationale for the curved temperature-rate plots observed for enzymes, independent of  
391 denaturation, and describes the temperature dependence of enzyme-catalyzed rates in the absence  
392 of denaturation by the difference in heat capacity between the enzyme substrate complex and the  
393 enzyme transition state species (Arcus et al. 2016). The three-state equilibration model (EM) (Daniel  
394 and Danson 2013) has also been suggested to explain the temperature dependence of enzyme-  
395 catalyzed rates in the absence of denaturation. EM introduces a reversible inactivated (not  
396 denatured) form of the enzyme ( $E_{inact}$ ) as an intermediate in rapid equilibrium with the active form  
397 ( $E_{act}$ ), which adds a thermal buffer effect that protects the enzyme from thermal inactivation.  
398 Another explanation invokes a tuning of surface mobility through alteration of regions spatially  
399 removed from the active site which affect the overall enzyme dynamics (Åqvist et al. 2017; Isaksen et  
400 al. 2016). Computer simulations and Arrhenius plots suggest that surface rigidity/flexibility outside  
401 the catalytic region affects the enthalpy/entropy balance. Key single distant mutations may disrupt  
402 surface hydrogen binding networks and alter the protein water surface interactions (Isaksen et al.  
403 2016) which may be the case with arginine substitutions in our study.

## 404 Conclusions

405 We have described the temperature optima and thermal denaturation profiles of three psychrophile-  
406 derived ADLs of the minimal Lig E-type. In the course of this work we determined that two of the three,  
407 Par-Lig and the structurally-characterized Psy-Lig did not exhibit marked psychrophilic properties,  
408 while the third had typical low-temperature characteristics such as low  $T_{opt}$  and low thermal stability.  
409 Sequence comparison and homology modeling identified surface-exposed patches with greater  
410 hydrophobicity in Vib-Lig, relative to homologs, which we suggest are relevant for the experimentally-  
411 observed psychrophilic properties.

412 Consistent with our observations for Vib-Lig and Vch-Lig, catalytic sites may be strictly conserved  
413 between homologs with different activity optima, meaning explanations for the markedly lower  $T_{opt}$  of  
414 Vib-Lig relative to Psy-Lig and Par-Lig cannot invoke specific increases in active-site flexibility. We hope

415 that future application of more sophisticated computational methods, coupled with specific  
 416 mutational studies may elucidate general principles imparting low-temperature activities which can  
 417 be transferred to commercially-relevant DNA ligases allowing us to tailor their activity optima in  
 418 biotechnological applications.

## 419 Figures

420 **Figure 1.** Temperature optimum of Psy-Lig, Par-Lig and Vib-lig by ligase activity assay. (a) Percentage  
 421 of ligated single-nicked substrate (b) Percentage of ligated cohesive substrate. Ligase activity was  
 422 quenched after 15 minutes at various temperatures and quantified as percentage ligation by the  
 423 intensity of the upper band relative to the sum of the two bands on the TBE-UREA gel. Ligase  
 424 concentration was 2.5  $\mu\text{M}$  for the nicked substrate, and 100  $\mu\text{M}$  for the cohesive substrate

425 **Figure 2.** Biophysical data. (a) Thermal unfolding monitored by DSC (b) Thermal stability measured by  
 426 thermofluor.

427 **Figure 3.** Amino acid sequence alignment comparing mature ATP-dependent ligases from  
 428 *Psychromonas* spp. strain SP041 (Psy-Lig), *Aliivibrio salmonicida* (Vib-Lig), *Pseudoalteromonas artica*  
 429 (Par-Lig) and *Vibrio cholera* (Vch-Lig). Identical residues are shaded with red and similar residues are  
 430 shown in red text. Spirals indicate  $\alpha$ -helices and arrows indicate  $\beta$ -strands. Boxed amino acids  
 431 represent conserved motifs of the nucleotidyltransferase enzymes. The DNA-binding elements of Lig  
 432 Es are boxed with dashed lines. Surface-exposed substitutions of basic to uncharged residues in Vib-  
 433 Lig are indicated by blue circles.

434 **Figure 4.** Sequence variability mapped onto molecular surface representations of Vib-Lig, Psy-Lig, Par-  
 435 Lig and Vch-Lig. The top and bottom panels are rotated 180° views, while the middle panel shows  
 436 melting temperature and substituted amino acids in selected positions for the four enzymes. Color  
 437 codes: Blue: positively charged residues; Green: polar residues; Orange: hydrophobic residues. Vib-Lig  
 438 Residue numbers are included for reference between the panels. There is an apparent correlation  
 439 between reduced thermostability and substitution from charged residues into more hydrophobic  
 440 ones.

441 **Figure 5.** Structure of Psy-Lig (top) and model of Vib-Lig (lower) colored surface charge. The surface  
 442 potential was generated using APBS (Dolinsky et al. 2007), with positively charged areas shown in  
 443 blue and negatively charged areas in red.

## 444 Tables

445 **Table 1.** Literature and experimental data showing host optimal growth temperature, ligase  
 446 temperature optimum for nick sealing and melting temperature.

| Ligase  | Species of origin                     | Optimal growth (°C) | T <sub>opt</sub> (°C) | T <sub>m</sub> (°C) |
|---------|---------------------------------------|---------------------|-----------------------|---------------------|
| Psy-Lig | <i>Psychromonas</i> spp. strain SP041 | 15 <sup>b</sup>     | 35 <sup>a</sup>       | 46 <sup>a</sup>     |
| Par-Lig | <i>Pseudoalteromonas artica</i>       | 10-15 <sup>c</sup>  | 35-40 <sup>a</sup>    | 53 <sup>a</sup>     |
| Vib-Lig | <i>Aliivibrio salmonicida</i>         | 15 <sup>d</sup>     | 20 <sup>a</sup>       | 30 <sup>a</sup>     |

447 <sup>a</sup> This study

448 <sup>b</sup> Groudieva et al (2003) (Groudieva et al. 2003)

449 <sup>c</sup> Khudary et al (2008) (Al Khudary et al. 2008)

450 <sup>d</sup> Egidius et al, (1986) (Egidius et al. 1986)

451

452 **Table 2.** Brief summary of extracted sequence features and characterization data for Psy-Lig, Vib-Lig,  
 453 Par-Lig and Vch-Lig, respectively.

|   | Psy-Lig | Vib-Lig | Par-Lig | Vch-Lig |
|---|---------|---------|---------|---------|
| <b>Sequence length</b>                      | 257     | 257     | 260     | 262     |
| <b>T<sub>opt</sub> (°C)</b>                 | 35      | 20      | 35-40   | -       |
| <b>T<sub>melt</sub> (°C)</b>                | 46.0    | 30.7    | 53.7    | -       |
| <b>Calculated pI</b>                        | 9.1     | 5.3     | 9.5     | 9.0     |
| <b>Net charge<sup>a</sup></b>               | +4      | -9      | +9      | +4      |
| <b>Polar residues<sup>b</sup> (%)</b>       | 35.8    | 29.2    | 37.3    | 34.0    |
| <b>Hydrophobic residues<sup>c</sup> (%)</b> | 41.3    | 42.8    | 39.2    | 39.7    |
| <b>Aromatic residues<sup>d</sup> (%)</b>    | 10.9    | 9.7     | 11.2    | 11.5    |
| <b>Gly (number and %)</b>                   | 17/6.6  | 24/9.3  | 20/7.7  | 23/8.8  |
| <b>Met (number and %)</b>                   | 7/2.7   | 8/3.1   | 3/1.2   | 6/2.3   |
| <b>Pro (number and %)</b>                   | 13/5.1  | 13/5.1  | 12/4.6  | 11/4.2  |
| <b>Arg (number and %)</b>                   | 12/4.7  | 11/4.3  | 12/4.6  | 18/6.9  |
| <b>Arg/(Lys+Arg)</b>                        | 0.40    | 0.39    | 0.39    | 0.53    |
| <b>(Leu+Ile)/(Leu+Ile+Val)</b>              | 0.76    | 0.70    | 0.65    | 0.78    |

- 454 - <sup>a</sup> Residues R, K, D and E  
 455 - <sup>b</sup> Residues G, S, T, Y, N, Q and C  
 456 - <sup>c</sup> Residues A, V, L, I, W, F, P and M  
 457 - <sup>d</sup> F, W and Y  
 458

459 **Table 3.** Summary of calculated intramolecular interactions for Psy-Lig, Vib-Lig, Par-Lig and Vch-Lig,  
 460 respectively

|  | Psy-Lig | Vib-Lig | Par-Lig | Vch-Lig |
|--|---------|---------|---------|---------|
| <b>PDB ID</b>  | 4d05    | Model   | Model   | Model   |
| <b>Resolution</b>                                    | 1.65 Å  | -       | -       | -       |
| <b>No. of residues in PDB file</b>                   | 257     | 256     | 257     | 250     |
| <b>No. of hydrogen bonds per residue</b>             | 0.759   | 0.715   | 0.778   | 0.816   |
| <b>No. SS<sup>e</sup> hydrogen bonds per residue</b> | 0.086   | 0.066   | 0.066   | 0.104   |
| <b>No. SM<sup>f</sup> hydrogen bonds per residue</b> | 0.202   | 0.133   | 0.175   | 0.180   |
| <b>No. MM<sup>g</sup> hydrogen bonds per residue</b> | 0.471   | 0.516   | 0.537   | 0.532   |
| <b>No. ion pairs &lt;4/&lt;6 Å</b>                   | 8/19    | 11/18   | 10/16   | 12/23   |
| <b>No. 2 membered networks &lt;4.0 Å</b>             | 6       | 5       | 6       | 6       |
| <b>No. 3 membered networks &lt;4.0 Å</b>             | 1       | 3       | 2       | 3       |

- 461 <sup>e</sup> SS, side-chain to side-chain hydrogen bonds.  
 462 <sup>f</sup> SM, side-chain to main-chain hydrogen bonds.  
 463 <sup>g</sup> MM, main-chain to main-chain hydrogen bonds.  
 464

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